DOCTORAL THESIS

Ecole Doctorale Biologie-Santé

Université de Lille

Specialty: Parasitology

Presented and defended by

Manasi SAWANT

April 26th 2022

Epigenetic and transcriptomic characterization of *Cryptosporidium* infection and their implication in digestive neoplasia development

Thesis Director:
Dr. Gabriela CERTAD (Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL), France; Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), France)

Thesis co-supervisor:
Dr. Sadia BENAMROUZ-VANNESTE (Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille, France/ FGES, Institut Catholique de Lille, France)

Jury Members

Pr. Isabelle FLORENT	Museum National d'Histoire Naturelle, Paris, France	**Reporter**
Dr. Hicham EL ALAOUI	Université Clermont Auvergne, Clermont Ferrand, France	**Reporter**
Pr. Tony LEFEBVRE	Université de Lille, Lille, France	**Jury President**
Pr. Sebastien MONCHY	Université du Littoral Côte d'Opale, Wimeraux, France	**Examiner**
Dr. Eric VISCOGLIOSI	Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL), Lille, France	**Examiner**
Dr. Gabriela CERTAD	Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL); GHICL, Lille, France	**Thesis Director**

U1019-UMR 9017-CIIL-Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, Université de Lille, CNRS, Inserm, CHU Lille, F-59000 Lille, France.

THÈSE DE DOCTORAT

Ecole Doctorale Biologie-Santé

Université de Lille

Spécialité: Parasitology

Présentée et soutenue publiquement par

Manasi SAWANT

April 26th 2022

Épigénétiques et génétiques de l'infection par *Cryptosporidium* et leurs impacts dans le développement des néoplasies digestives

Directrice de thèse:
Dr. Gabriela CERTAD (Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL), France; Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), France)

Co-encadrante de thèse:
Dr. Sadia BENAMROUZ-VANNESTE (Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille, France/ FGES, Institut Catholique de Lille, France)

Membres du Jury

Pr. Isabelle FLORENT	Museum National d'Histoire Naturelle, Paris, France	**Rapporteur**
Dr. Hicham EL ALAOUI	Université Clermont Auvergne, Clermont Ferrand, France	**Rapporteur**
Pr. Tony LEFEBVRE	Université de Lille, Lille, France	**Président du Jury**
Pr. Sebastian MONCHY	Université du Littoral Côte d'Opale, Wimeraux, France	**Examinateur**
Dr. Eric VISCOGLIOSI	Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL), Lille, France	**Examinateur**
Dr. Gabriela CERTAD	Centre d'Infection et d'Immunité de Lille (CIIL)/Institut Pasteur de Lille (IPL); GHICL, Lille, France	**Directrice de thèse**

U1019-UMR 9017-CIIL-Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, Université de Lille, CNRS, Inserm, CHU Lille, F-59000 Lille, France.

“In the history of Science, we
often find that the study of
some natural phenomenon
has always been the starting point
in the development of a
new branch of knowledge”

- Nobel laureate Dr. C. V. Raman

Acknowledgements

This thesis project was financially supported by Institut Pasteur de Lille (IPL), Institut national de la santé et de la recherche médicale (Inserm), Centre national de la recherche scientifique (CNRS), the Catholique university of Lille and the University of Lille.

I would like to thank the Ecole Doctoral de Biologie et Santé de Lille (EDBSL), the University of Lille, for financial support during the thesis.

The past three years have not only been a source of professional but also immense personal growth. I would like to express my gratitude to all the people mentioned here who have directly or indirectly enriched my Ph.D. journey.

To the Jury members,

Pr. Isabelle FLORENT

I am honored to have you as a jury member for my thesis. Your expertise in the field of parasitology, particularly, the development of unicellular eukaryotes would be very insightful in critically reviewing our work. I believe you shall be glad to encounter a different aspect of parasite development through our research.

Dr. Hicham EL ALAOUI

I am equally honored to have you as a jury member. I thank you for critically reviewing my work since the first year of my thesis. I believe you will acknowledge my progress and continue to guide me with your expertise as a reporter of the thesis.

Pr. Tony LEFEBVRE

I wish to express my gratitude for agreeing to be one of my jury members. I believe that apart from guiding me since the first year of my thesis, you would be pleased to see the advancement made by our team concerning *Cryptosporidium* and cancer. Indeed to benefit from your profound knowledge in the field of cancer research would be highly appreciated.

Pr. Sebastian MONCHY

My sincere regards for accepting to be part of my thesis jury member. It would be an honor to share my transcriptomics results with you considering it is your field of expertise.

Acknowledgements

Dr. Eric VISCOGLIOSI

I would like to express my deepest gratitude to you for welcoming me to your laboratory. It all started with your spontaneous reply to my email asking you for a Ph.D. position back in July 2017. Despite your responsibilities as a team leader, the enthusiasm with which you were capable of having scientific discussions has been extremely valuable to me. I would sincerely like to thank you for all the knowledge, skills, and advice you have shared over the past three years. Thanks to your contagious spontaneity and encouragement, today I feel confident enough to start a journey as a researcher.

Dr. Gabriela CERTAD

I would like to thank you for giving me the opportunity and introducing me to *Cryptosporidium*. I thank you for always appreciating my input and ideas to develop the thesis project. It gave me the confidence to carry out my work independently. Of course, your scientific aptitude and critical advice have helped me crucially in writing the research articles and the thesis. I hope to have inculcated at least an ounce of your serene and composed approach toward life, which is truly inspirational.

Dr. Sadia BENAMROUZ-VANNESTE

I would equally like to thank you for providing me with an occasion to be introduced to the world of parasitology through *Cryptosporidium*. I remember our first encounter when you relentlessly motivated and prepared me for the international contest to obtain the Ph.D. grant. Since then, you have been a quintessential part of my Ph.D. journey. I am grateful for everything from providing opportunities to co-supervise students to encouraging me to be scientifically independent. You never hesitated in helping me thanks to your selfless nature which is very admirable.

Acknowledgements

To other members of the laboratory and collaborators,

I am immensely grateful to the entire team of Ecology and physiopathology of intestinal protozoa (ECOPHIP) and other collaborative members of the team for their patience and support during my thesis.

Dr. Magali Chabé who is the constant and strong support of the team has always been there to answer my queries regarding genomics and transcriptomics. Her passion for research is immensely motivational and I thank her for that. I would like to thank **Nausicaa Gantois** for being the most energetic member of the team. Her charismatic and sympathetic personality has always been very welcoming and made her the go-to person. **Jeremy Desramaut**, the latest member to join the team, I thank you for making the tremendous effort to communicate with me in English. I highly appreciate it. I would also like to thank **Karine Guyot** and **Dr. Dionigia Meloni** for being on the team during the initial days of my Ph.D. I thank them for introducing and teaching me about the different techniques utilized in the lab to study *Cryptosporidium*. I would also like to thank **Dr. Martha Baydoun** for introducing me and helping me establish my very first explant culture experiment in the lab. I am thankful to have great colleagues like **Dr. Salma Khaled** and **Marianne Darras**. Indeed we met each other as colleagues but it took no time to become friends. I am grateful for your friendship. I am also thankful to all the interns with whom I got the opportunities to interact or supervise during my Ph.D

I would also like to thank several members of the animal facility for their help with all the animal experiments. I thank you **Anthony Mouray** for helping me with the animal experiments, especially amidst the pandemic. I would like to thank **Sophie Salomé-Desnoulez** and **Elisabeth Werkmeister** from the bioimaging platform for their assistance and guidance in my confocal microscopy experiments.

I would like to thank members of the St Vincent de Paul Hospital. I thank **Erika Duval** for introducing me to the techniques of histopathology and providing histological slides for various animal experiments. I am grateful to **Prof. Colette Creusy** for giving her critical analysis of all the histological examinations. I wish to have gotten more opportunities to learn about histopathology under your supreme expertise.

I would like to thank members of the team Transcriptomics and Applied Genomics at Institute Pasteur of Lille, **Peggy Bouquet**, for assisting with the transcriptomics project, especially for

Acknowledgements

the microarray experiments. I am extremely thankful to **Dr. David Hot** for helping me in analyzing the microarray data and being tremendously patient in teaching me the IPA software.

I am undoubtedly grateful to **Dr. René Wintjens** for helping me bring my idea of introducing superimposition and 3D homology in the epigenetics project into reality. Thank you for all your precise guidance, only to realize, that I wish I had more time to learn about structural biology from you.

Undeniably, I would like to thank my friends outside my professional circle who have enriched my stay in Lille in one way or the other. Abhishek, Rakesh, Shiva, Natalie, Tommaso, Konstantinos, Georgia, and Sarah, I will always cherish the time spent with all of you.

Certainly, this Ph.D. journey holds no significance without thanking my friends and family back home who motivated and encouraged me to take up this opportunity to pursue my Ph.D. in a foreign country. Rutuja and Radhika, I cannot thank you enough for being by my side for more than 10 years. I think the distance just made our bond stronger and your words of courage and wisdom have been my source of inspiration. My deepest gratitude to my entire extended family who has supported me as dearly and as compassionately as my parents. Indeed, this journey would not have been the same without you Ajinkya. Looking forward to a lifetime of togetherness and a journey full of new adventures.

Finally, my heartfelt gratitude to my parents and my younger sister, without whom this journey wouldn’t have begun, for their unconditional support in each of my decisions and for giving me the motivation to always go a little further.

Publications

- **Sawant, M.**; Benamrouz-Vanneste, S.; Meloni, D.; Gantois, N.; Guyot, K.; Creusy, C.; Duval, E.; Wintjens, R.; Weitzman, J.; Chabé, M.; Viscogliosi, E.; Certad, G. **Putative SET-domain methyltransferases in *Cryptosporidium parvum* and histone methylation during infection.** article submitted to the journal "Virulence" and is under revision.

- Guilavogui, T.; Gantois, N.; Even, G.; Desramaut, J.; Dautel, E.; Denoyelle, C.; Cissé, F.I.; Touré, S.C.; Kourouma, B.L.; **Sawant, M.**; Chabé, M.; Certad, G.; Viscogliosi, E. **Detection, Molecular Identification and Transmission of the Intestinal Protozoa *Blastocystis* sp. in Guinea from a Large-Scale Epidemiological Study Conducted in the Conakry Area**. *Microorganisms* **2022**, *10*, 446. https://doi.org/10.3390/microorganisms10020446

- **Sawant, M.**; Benamrouz-Vanneste, S.; Mouray, A.; Bouquet, P.; Gantois, N.; Creusy, C.; Duval, E.; Mihalache, A.; Gosset, P.; Chabé, M.; Hot, D.; Viscogliosi, E.; Certad, G. **Persistent *Cryptosporidium parvum* Infection Leads to the Development of the Tumor Microenvironment in an Experimental Mouse Model: Results of a Microarray Approach**. *Microorganisms* **2021**, *9*, 2569. https://doi.org/10.3390/microorganisms9122569

- Landman, W. J. M.; Gantois, N.; **Sawant, M.;** Majoor, F. A.; Eck E, J. H. H. van.; Viscogliosi, E. **Prevalence of trichomonads in the cloaca of wild wetland birds in the Netherlands**, *Avian Pathology* **2021**, 50:6, 465-476. https://doi.org/10.1080/03079457.2021.1967876

- Khaled, S.; Gantois, N.; Ayoubi, A.; Even, G.; **Sawant, M.**; El Houmayraa, J.; Nabot, M.; Benamrouz-Vanneste, S.; Chabé, M.; Certad, G.; El Safadi, D.; Dabboussi, F.; Hamze, M.; Viscogliosi, E. ***Blastocystis* sp. Prevalence and Subtypes Distribution amongst Syrian Refugee Communities Living in North Lebanon**. *Microorganisms* **2021**, *9*, 184. https://doi.org/10.3390/microorganisms9010184

- Certad, G.; Zahedi, A.; Gantois, N.; **Sawant, M.**; Creusy, C.; Duval, E.; Benamrouz-Vanneste, S.; Ryan, U.; Viscogliosi, E. **Molecular Characterization of Novel *Cryptosporidium* Fish Genotypes in Edible Marine Fish**. *Microorganisms* **2020**, *8*, 2014. https://doi.org/10.3390/microorganisms8122014

- **Sawant, M.**; Baydoun, M.; Creusy, C.; Chabé, M.; Viscogliosi, E.; Certad, G.; Benamrouz-Vanneste, S. ***Cryptosporidium* and Colon Cancer: Cause or Consequence?** *Microorganisms* **2020**, *8*, 1665. https://doi.org/10.3390/microorganisms8111665

- Khaled, S.; Gantois, N.; Ly, A.T.; Senghor, S.; Even, G.; Dautel, E.; Dejager, R.; **Sawant, M.**; Baydoun, M.; Benamrouz-Vanneste, S.; Chabé, M.; Ndiaye, S.; Schacht, A.-M.; Certad, G.; Riveau, G.; Viscogliosi, E. **Prevalence and Subtype Distribution of *Blastocystis* sp. in Senegalese School Children**. *Microorganisms* **2020**, *8*, 1408. https://doi.org/10.3390/microorganisms8091408.

- **Sawant, M.**; Benamrouz, S.; Chabé, M.; Guyot, M.; Costa, M.; Favennec, L.; Gargala, G.; Viscogliosi, E.; Certad, G. **La cryptosporidiose et son impact en santé publique.** *Revue de Biologie Médicale* **2020**, 352.

Communications and Accolade

Poster Presentations

- **Sawant M *et al*,** 2021. Symposium day, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (28th September 2021). "Characterization of histone lysine methylation regulators during *Cryptosporidium parvum* infection". **Prize: Won 3rd place in poster presentation competition**.

- **Sawant M *et al*,** 2021. 13th European Multicolloquium of Parasitology, Belgrade, Serbia, (12th – 16th October 2021). "First characterization of the role of epigenetics in the dynamics of *Cryptosporidium parvum* infection".

- **Sawant M *et al*,** 2021. 13th European Multicolloquium of Parasitology, Belgrade, Serbia, (12th – 16th October 2021). "*Cryptosporidium parvum* infection and colon cancer: Results of microarray approach".

- Khaled, S.; Gantois, N.; Ayoubi, A.; Even, G.; **Sawant, M *et al*,** 2021. 13th European Multicolloquium of Parasitology, Belgrade, Serbia, (12th – 16th October 2021). Molecular epidemiology of blastocysti amongst Syrian refugee communities living in North Lebanon".

- Landman, W J M.; **Sawant, M** *et al,* 2021. 13th European Multicolloquium of Parasitology, Belgrade, Serbia, (12th – 16th October 2021). "Molecular epidemiology of trichomonads in wild wetland birds in the Netherlands".

- Khaled, S.; Gantois, N.; Ayoubi, A.; Even, G.; **Sawant, M *et al*,** 2021. 3rd International Blastocystis Conference. (2nd – 4th June 2021). "Prevalence and subtype distribution of *Blastocystis* sp. amongst Syrian refugee communities living in North Lebanon". (online conference).

- **Sawant M *et al,*** 2019. Symposium day, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (30th September 2019). "Is *Cryptosporidium parvum* able to induce intestinal neoplasia *in vitro*?"

- **Sawant M *et al,*** 2019. 7th International *Giardia* and *Cryptosporidium* Conference, Rouen, France. (24th – 26th June 2019). " Is *Cryptosporidium parvum* able to induce intestinal neoplasia *in vitro*?"

Oral Presentations

- **Sawant M**. 2022. 'Work in progress' symposium, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (28th January 2022). " Persistent *Cryptosporidium parvum* infection leads to the development of tumor microenvironment in an experimental mouse model : Results of microarray approach".

- **Sawant M.** 2021. 15th International Workshop on Opportunistic Protists, České Budějovice, Czech Republic. (15th – 17th June 2021) "First study to characterize the role of epigenetics in the biology of the enteric parasite *Cryptosporidium parvum"*. (online conference).

- **Sawant M.** 2021. 'Work in progress' symposium, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (16th April 2021). "Characterization of the parasite and host epigenetic mechanisms involved during *Cryptosporidium parvum* infection".

Communications and Accolade

- **Sawant M**. 2020. 20th André Verbert Day, Lille, France (27th November 2020). "Characterization of the host and parasite epigenetic mechanisms involved during *Cryptosporidium parvum* infection". (online conference).

- **Sawant M**. 2020. 'Work in Progress' symposium, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (21st February 2020). " Characterization of the molecular signature of *Cryptosporidium parvum* induced digestive cancer".

Training courses and student co-supervision

- Bioinformatics courses
  - High-throughput sequencing data analysis training - Plateforme de bioinformatique et de bioanalyze de Lille, Lille, France.
    Module 1 - DNA analysis (7th - 8th March 2019).
    Module 2 – Variant analysis (1st – 3rd April 2019).
    Module 3 – Omics data analysis (20th – 21st May 2019).
    Module 4 – Metagenomics (20th – 22nd November 2019).

  - Cytoscape for visualization and analysis of biological networks - Plateforme de bioinformatique et de bioanalyze de Lille, Lille, France. (23rd – 24th January 2020).

- Methodological and interdisciplinary training
  - Animal experimentation training – University of Lille, CHRU de Lille, Lille, France. (8th November – 13th December 2019).

- Scientific seminars - Centre of Infection and Immunity of Lille, Lille, France
  - The fascinating biology of enteric viruses (Speaker -Dr. Christiane Wobus, University of Michigan, USA).
  - Studying host-bacterial interactions in health and diseases using microfluidics and organ on chip biomimetic systems (Speaker - Dr. Alexandre Grassart, Pasteur Institute Shanghai, China and Lille, France).
  - Response of the human gut and saliva microbiome to urbanization in Cameroon (Speaker – Dr. Laure Segurel, Muséum national d'Histoire naturelle (MNHN) et de l'Université de Paris, France).

- Non-scientific courses
  - French language course for Ph.D. students – University of Lille, Lille, France. (semester I and II 2019).

- Student co-supervision
  - Victoria Questel (Co-supervision with Dr. Sadia Benamrouz-Vanneste): 2nd year Bachelor of Science (Biology) degree student, Catholique University of Lille. The academic year 2019-20. The total duration of the internship: 6 weeks.

  - Clara Cheung (Co-supervision with Dr. Sadia Benamrouz-Vanneste): 1st year Master of Science (Biology and health) degree student, University of Lille. The academic year 2019-20. The total duration of the internship: 3 weeks.

  - Mathieu Corbin (Co-supervision with Dr. Sadia Benamrouz-Vanneste): 3rd year Bachelor of Science (Biology) degree student, Catholique University of Lille. The academic year 2020-2021. The total duration of the internship: 6 weeks.

  - Constance Denoyelle (Co-supervision with Dr. Eric Viscogliosi): 1st year Master of Science (Biology) degree student, University of Lille. The academic year 2021-2022. The total duration of internship: 6 weeks.

Abstract

Cryptosporidium is an Apicomplexan parasite that infects the gastrointestinal tract of a variety of vertebrates including humans, being diarrhea the main clinical manifestation. Diarrhoeal illness can be severe or even fatal, especially in immunocompromised hosts. More strikingly, experimental and epidemiological evidence suggests a causal link between *Cryptosporidium* infection and digestive cancer. Indeed, the development of invasive digestive adenocarcinoma has been reported in an experimental model of dexamethasone-treated SCID mice. Even if this parasite was declared a public health problem by the World Health Organization (WHO) in 2006, its pathogenesis remains poorly understood. For all these reasons it seemed interesting to carry out this thesis project focused on the study of interactions between *Cryptosporidium* and the host to explore the contribution of the parasite to cancer development. Thus, we decided to articulate the research around two main objectives: 1) To study the role of epigenetics on the interaction between *Cryptosporidium* and its host, and 2) To investigate signaling pathways involved in *Cryptosporidium parvum* induced digestive cancer. Firstly, considering that epigenetics has an important role in transcription regulation, we investigated histone lysine methylation, as a dynamic epigenetic modification during the complex life cycle of the parasite. Initially, we performed *in silico* analysis to identify potential histone lysine methyltransferases (HKMTs) of *Cryptosporidium*. Primary sequence alignment and phylogenetic analysis allowed the identification of putative *C. parvum* HKMTs and their substrate specificities. By homology modeling, we predicted three structurally active HKMTs namely CpSET1, CpSET2, and CpSET8, and their functional significance was justified by observing histone lysine methylations such as H3K4Me3, H3K36Me3, and H4K20Me3 during intracellular development of *C. parvum*. We cloned and produced the putative HKMT, CpSET8 into a bacterial system, and via an *in vitro* activity assay we proved the existence of an active HKMT in the parasite. Moreover, host lysine methylation events were also explored and the results highlighted the inherent potential of the parasite to exploit the host epigenetic regulation to its advantage. This is the first study to highlight the existence of epigenetic mechanisms regulating the *Cryptosporidium* life cycle and its interaction with the host. Secondly, using the mouse model of cryptosporidiosis successfully developed in the ECOPHIP laboratory, a microarray approach was performed to compare *C. parvum* infected animals to non-infected controls. Results of this study showed that *C. parvum* can evade the host's innate immune response by resisting the upregulated expression of Interferon-γ (*IFNγ)*-stimulated genes, and downregulating the expression of α-defensins giving rise to chronic inflammation. Systematic

Abstract

inflammation may contribute to the *C. parvum*-induced immunosuppressive tumor microenvironment (TME) characterized by the presence of cancer-associated fibroblasts, myeloid-derived suppressor cells, tumor-associated macrophages, and extracellular matrix components. This is the first description of the alteration in the gene expression profile associated with the *C. parvum*-induced neoplasia. Finally, since the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway was also modulated according to the transcriptomic results, and PI3K is a signaling kinase implicated in cytoskeletal reorganization, which seems to be pivotal in the process of *C. parvum*-induced neoplasia, we decided to study in more depth the implication of this pathway. Results of immunohistochemistry and western blot confirmed the activation of this signaling cascade in *C. parvum*-infected epithelial cells. All these data showed that the mechanisms that contribute to the pathogenicity of the parasite and the induction of digestive neoplasia are multifactorial and involve both parasite and host factors.

Résumé

Cryptosporidium est un parasite Apicomplexa infectant le tractus gastro-intestinal d'un grand nombre de vertébrés dont l'homme. La diarrhée est la principale manifestation clinique. Elle peut s'avérer grave, voire mortelle chez les individus immunodéprimés. De plus, de nombreuses études expérimentales et épidémiologiques suggèrent qu'il existe un lien de causalité entre l'infection par *Cryptosporidium* et le cancer digestif. En effet, il a été rapporté que ce parasite pouvait induire le développement d'adénocarcinomes digestifs invasifs chez un modèle expérimental de souris SCID traitées à la dexaméthasone. Bien qu'il ait été considéré depuis 2006, par l'Organisation Mondiale de la Santé (OMS) comme étant un problème de santé publique, sa pathogénie reste mal connue. Pour toutes ces raisons, il nous a semblé intéressant, dans le cadre de ce projet de thèse, d'étudier les interactions entre *Cryptosporidium* et son hôte afin d'explorer son rôle dans le développement du cancer. Ce travail s'articule donc autour de deux principaux objectifs : 1) Etudier le rôle de l'épigénétique dans les interactions entre *Cryptosporidium* et son hôte, et 2) Etudier les voies de signalisation impliquées dans le développement du cancer digestif induit par *Cryptosporidium parvum*. Dans un premier temps, considérant que l'épigénétique joue un rôle important dans la régulation de la transcription, nous avons étudié la méthylation des résidus lysine des histones, au cours du cycle biologique du parasite. Une première analyze *in silico* a permis d'identifier les histone lysine méthyltransférases (HKMTs) potentielles de *Cryptosporidium*. Ensuite, l'alignement des séquences primaires et l'analyze phylogénétique ont permis d'identifier les HKMTs putatives de *C. parvum* et leurs spécificités de substrat. De plus, nous avons également pu prédire par modélisation, l'existence de trois HKMTs structurellement actives, à savoir CpSET1, CpSET2 et CpSET8. Leur rôle fonctionnel a été justifiée par l'observation de la méthylation de lysine d'histones telles que H3K4Me3, H3K36Me3 et H4K20Me3 pendant le développement intracellulaire de *C. parvum* nous avons réussi via des clonages et des tests d'activité *in vitro*, à montrer l'existence d'une HKMT CpSET8 active. De plus, les événements de méthylation de la lysine de l'hôte ont également été explorés et les résultats ont mis en évidence le potentiel du parasite à exploiter la régulation épigénétique de l'hôte à son avantage. C'est la première étude mettant en évidence l'existence de mécanismes épigénétiques régulant le cycle biologique de *Cryptosporidium*. Dans un second temps, nous avons réalisé, toujours sur le même modèle murin, une étude transcriptomique qui a montré que *C. parvum* est capable d'échapper à la réponse immunitaire innée de l'hôte en résistant à la surexpression des gènes stimulés par l'IFNγ, et en régulant à la baisse l'expression des α-défensines, donnant lieu à une inflammation chronique. Cette inflammation systématique peut donc contribuer au

Résumé

microenvironnement tumoral (TME) immunosuppressif induit par *C. parvum*. Il s'agit de la première description de l'altération du profil d'expression génétique associé à la néoplasie induite par *C. parvum*. Enfin, cette étude a également permis de mettre en évidence le fait que la voie de signalisation phosphatidylinositol 3-kinase (PI3K)/AKT était également modulée et sachant que PI3K est une kinase de signalisation impliquée dans la réorganisation du cytosquelette lors de l'infection par *C. parvum*, nous avons décidé d'étudier plus en profondeur l'implication de cette voie dans l'induction du cancer par ce parasite. Les résultats des analyzes biochimiques et immunohistochimiques, ont confirmé l'activation de cette cascade de signalisation dans les cellules épithéliales infectées par *C. parvum*. Toutes ces données plaident en faveur d'une induction multifactorielle des néoplasies digestives par le parasite impliquant des facteurs liés à la fois au parasite et à l'hôte

List of abbreviations

ApiAP2	Apicomplexan Apetala 2
AdoMet	S-adenosyl-L-methionine
CDC	Centers for Disease Control and Prevention
C. canis	*Cryptosporidium canis*
C. cuniculus	*Cryptosporidium. cuniculus*
C. felis	*Cryptosporidium felis*
C. hominis	*Cryptosporidium hominis*
C. meleagridis	*Cryptosporidium. meleagridis*
C. muris	*Cryptosporidium muris*
C. parvum	*Cryptosporidium parvum*
C. ubiquitum	*Cryptosporidium. ubiquitum*
C. viatorum	*Cryptosporidium viatorum*
CCL20	C-C motif chemokine ligand 20
CXCL12	C-X-C Motif Chemokine Ligand 12
CDSs	coding DNA sequences
CAFs	Cancer-associated fibroblasts
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
DEFA2	Defensin-α-2
DEFA2	Defensin-α-2
DEFA4	Defensin-α-4
DEGs	differentially expressed genes
EMT	Epithelial-Mesenchymal Transition
FGF7	Fibroblast Growth Factor 7
GBPs	Guanylate binding proteins
GCN5	general control nonderepressible-5
HKMTs	Histone lysine methyltransferases
H3 K4	Lysine 4 of histone 3
H3 K9	Lysine 9 of histone 3
H3 K36	Lysine 36 of histone 3
H3K4Me2	di-methylation of H3K4

List of abbreviations

H3K4Me3	tri-methylation of H3K4
H3K9Me3	tri-methylation of H3K9
H3K27Me3	tri-methylation of H3K27
H3K36Me3	tri-methylation of H3K36
H4K20Me1	mono-methylation of H4K20
H4K20Me2	di-methylation of H4K20
H4K20Me3	tri-methylation of H4K20
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HMG	High Mobility Group
IPA	Ingenuity pathway analysis
IECs	Intestinal epithelial cells
IL-1β	interleukin-1-beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL18	Interleukin-18
IL33	Interleukin 33
IFNγ	Interferon-gamma
IGF	Insulin-like growth factor
IIGP1	Interferon-induced GTPase 1
IDO1	Indoleamine 2,3-Dioxygenase 1
ILC	Innate lymphoid cells
JmjC	Jumonji C-terminal
KDMs	(Histone) lysine demethylases
LSD	Lysine-specific demethylases
LRP5	Low-density lipoprotein receptor-related protein-5 (*LRP5*)
MDSCs	Myeloid-derived suppressor cells
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
ncRNA	non-coding RNA
NK	Natural killer

List of abbreviations

P. falciparum	*Plasmodium falciparum*
PHD	Plant homeodomain
PI3K	Phosphatidylinositol 3-kinase
PRMTs	Protein (histone) arginine-methyltransferases
PTGS2	Prostaglandin- Endoperoxide Synthase 2
SCID	Severe Combined Immunodeficiency
SET	[**S**u(var)3-9, **E**nhancer-of-zeste, **T**rithorax]
SNVs	Single nucleotide variants
SLC7A8	Solute Carrier Family 7 Member 8
STAT1	Signal transducer and activator of transcription 1
SSP1	Secreted phosphoprotein 1
T. gondii	*Toxoplasma gondii*
TAMs	Tumor-associated macrophages
TME	Tumor microenvironment
TEEGR	*Toxoplasma* E2F4-associated EZH2-inducing Gene Regulator
TgPrx1	Typical 2-cys peroxiredoxin-1
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
WHO	World Health Organization

List of figures and tables

List of figures

List of tables

Table of Content

Introduction

1. Introduction

Cryptosporidium is an intracellular protozoan parasite that infects the gastrointestinal epithelia of a wide variety of vertebrate hosts such as mammals, birds, reptiles, amphibians, and fish (Feng Y et al. 2018). *Cryptosporidium* belongs to the phylum Apicomplexa and even if it was considered for a long time, to be an atypical coccidian, it has been speculated currently that the parasite is more closely related to gregarine parasites according to molecular and biological similarities with this group (Ryan U et al. 2016). The field of parasitology is always benefiting from the latest discoveries associated with this pathogen which is a leading cause of diarrheal disease in young children and immunocompromised individuals. Thus, I would like to present in this section very recent breakthroughs in this field including updates in terms of description of new species, discoveries about the life cycle, host immune response and transmission, and epidemiology.

Update on Cryptosporidium *species*

The development of molecular detection techniques has assisted in the identification of a large number of *Cryptosporidium* species and genotypes and in turn strengthen our understanding of their potential for zoonotic transmission. To date, 44 *Cryptosporidium* species and more than >120 genotypes have been recognized. Out of these 19 different species and four genotypes have been reported in humans (Ryan U et al. 2021). Some species such as *Cryptosporidium meleagridis, Cryptosporidium canis, Cryptosporidium felis, Cryptosporidium viatorum,* and *Cryptosporidium muris* are more commonly identified in humans in developing countries, whereas others such as *Cryptosporidium ubiquitum, Cryptosporidium cuniculus,* and chipmunk genotype I are predominantly seen in developed countries (Yang X et al. 2021). Even then, *Cryptosporidium hominis* and *C parvum* are mainly responsible for 90 % of human cryptosporidiosis (Ryan U et al. 2021).

The life cycle of Cryptosporidium

Until today it is accepted that *Cryptosporidium* infection begins with the ingestion by a susceptible host of the environmentally resistant, sporulated oocyst, containing 4 sporozoites. Unlike other Apicomplexa, its development takes place in a single host within three days. As a typical protist belonging to this phylum, it relies on asexual and sexual reproduction. Interestingly, with the availability of current technologies such as CRISPR-cas9, the parasite

can be genetically manipulated to study its developmental cycle (Vinayak S et al. 2015) (Tandel J et al. 2019). Using this tool it has been proven that the development of sexual stages is an obligation to maintain the infection in the host (Tandel J et al. 2019). Briefly, the sporozoite upon invasion of the intestinal epithelial cells (IECs) undergoes asexual replication to develop type I meront containing 8 nuclei. The maturation of type I meronts gives rise to merozoites which are capable of reinfecting the host and develop into type II meronts containing 4 nuclei. The merozoites emerging from this meront stage are known to be committed to developing into the sexual stages both male and female. The male gamont undergoes four rounds of synchronous nuclear division to produce 12 to 16 gametes whereas the female gamete remains haploid until being fertilized to develop into a future oocyst. However, due to the asynchronous nature of its life cycle, the parasite alternates between asexual and sexual stages (Figure 1).

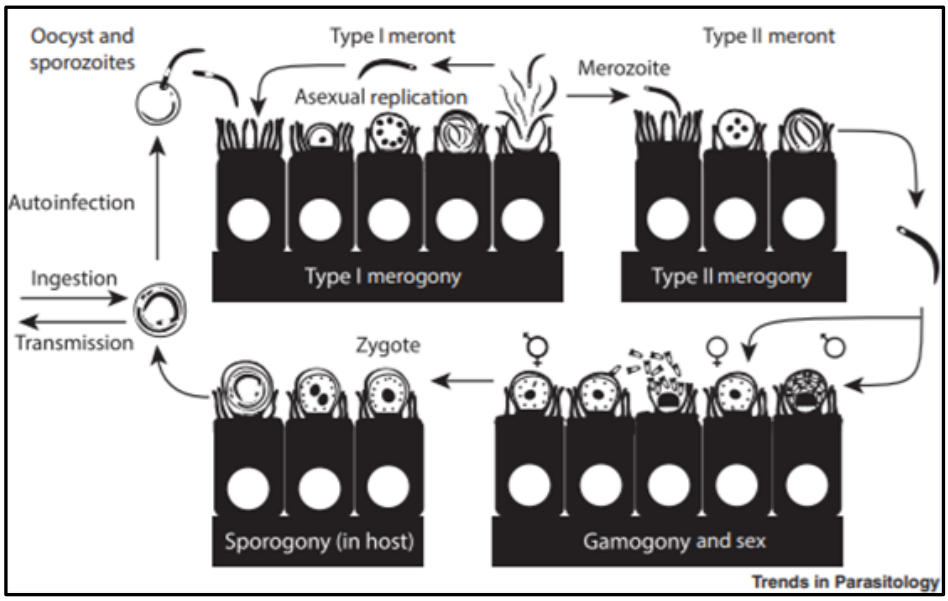


Figure 1. *Cryptosporidium* life cycle. Schematic representation of the *C. parvum* life cycle that highlights the presence of Type II merogony as an obligate step towards male and female gamete formation. The development of sexual stages and an obligate progression to sex is essential for the parasite to maintain the infection in the host. (Image was adapted from (Dumaine JE et al. 2019)).

Introduction

However, a brief update on the latest development of the study of the life cycle suggests that there is potentially no evidence of the existence of a morphologically distinct intermediate stage of 4N type II meronts. Recently, with the use of genetically engineered strains of the parasite and live-cell imaging, *Cryptosporidium* has been speculated to undergo three generations of asexual replication, followed by a single generation of sexual stages that is independent of environmental stimuli. The study demonstrates the direct development of gametes from type I meront (Figure 2) (English ED et al. 2021).

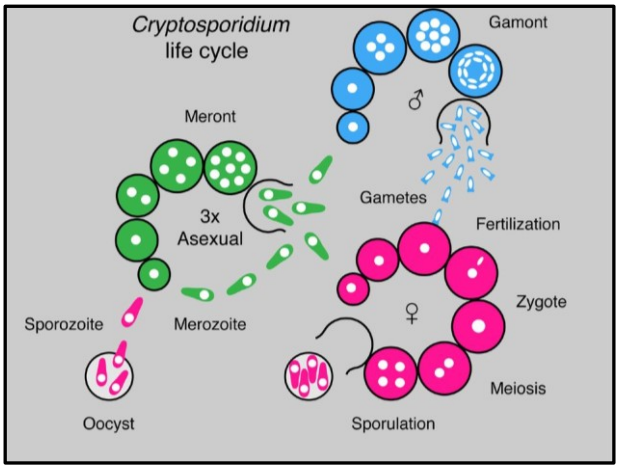


Figure 2. The potential existence of *Cryptosporidium* life cycle without the type II merogony. Merozoites emerging from type I meronts are committed to either asexual (green) or sexual fate. When sexually committed they can give rise to either male (blue) or female (pink) gametes. (Image was adapted from (English ED et al. 2021)).

Thus, the identification of molecular marks essential for proliferation and differentiation potentially involved in the regulation of the parasite life cycle could contribute to the understanding of the pathogenicity of *Cryptosporidium*, and be exploited for therapeutic purposes in near future. In this respect, in the frame of the thesis, we aimed to study the epigenetic regulators which might have a significant role in the development of the parasite and infection.

Introduction

Host immune response to Cryptosporidium

The immune response generated during the replication of the parasite contributes to the development of severe, watery diarrhea in infected individuals. Immunity to *Cryptosporidium* involves both innate as well as adaptive immune responses (Figure 3). In recent years, several studies have highlighted the importance of innate immunity to control infection. Innate resistance to *Cryptosporidium* is especially dependent on the production of a cytokine, interferon-gamma (IFNγ). IFNγ secreted by innate lymphoid cells (ILCs) and natural killer (NK) cells are essential for controlling the infection by increasing the expression of many interferon-stimulated genes such as Indoleamine 2,3-Dioxygenase 1 (IDO1), an enzyme that catabolizes tryptophan, thus limiting parasite growth in the IECs (Choudhry N et al. 2009). Recently, enterocytes have also been identified to promote IFNγ production in ILCs which in turn restricts the growth of *Cryptosporidium* specifically invading enterocytes (Gullicksrud JA et al. 2022). Moreover, transgenic parasites generated with the use of CRISPER-driven genome engineering have demonstrated that IFNγ is essential for early control of the infection whereas T-cells are critical for clearance of the parasite (Sateriale A et al. 2019). Recently, the role of the inflammasome in controlling parasite infection has also been showcased. *Cryptosporidium* triggers the enterocyte-intrinsic inflammasome which results in the release of the proinflammatory cytokine, interleukin-18 (IL18) (Sateriale A et al. 2021). Alongside, anti-microbial peptides secreted by Paneth cells, and secretory epithelial cells, also contribute to the control of the infection (Laurent F and Lacroix-Lamandé S 2017).

The intestinal inflammatory response seems to mediate diarrhea. Pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1β (IL1β) have been predicted to hinder the intestinal permeability (Sablet T et al. 2016). Both TNFα and IL1β are stimulators of prostaglandin production. Although these cytokines themselves do not contribute to diarrhea, prostaglandins may contribute to diarrhea during infection by altering chloride secretion (Borad A and Ward H 2010).

Introduction

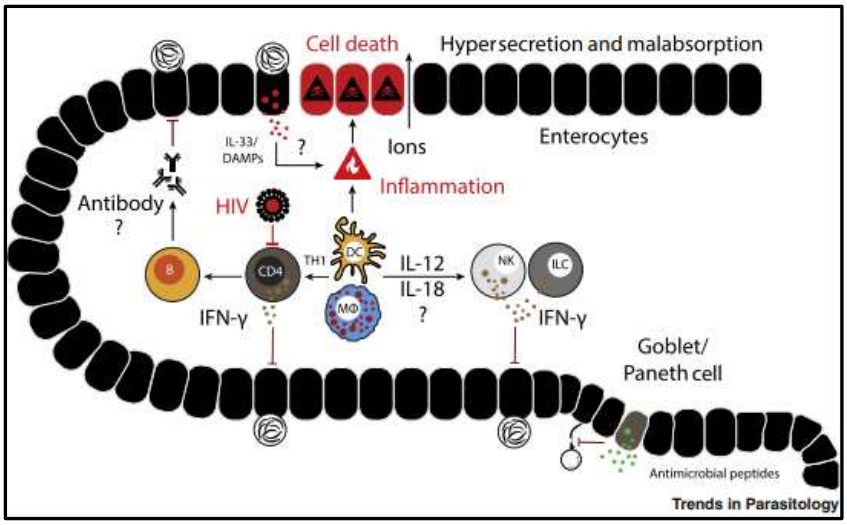


Figure 3. *Cryptosporidium* and host interactions. Overview of cells and molecules of adaptive and innate immunity involved in restriction of *Cryptosporidium* infection of the intestinal epithelial crypt. (Image was adapted from (Dumaine JE et al. 2019)).

Progress in terms of understanding o transmission and epidemiology

Cryptosporidium causes self-limited watery diarrhea in immunocompetent individuals but has devastating effects in those who are immunocompromised. Recently, a cohort study involving 22,500 children from Africa and Asia revealed that *Cryptosporidium* is the second cause of childhood diarrhea leading to mortality (Kotloff KL et al. 2013; Levine MM et al. 2019). The parasite is transmitted by the oral-fecal route and as a consequence, a large majority of *Cryptosporidium* infections have been documented to be the result of waterborne outbreaks. Between 2011 and 2016, 239 waterborne outbreaks were reported in Europe, Australia, and North America (Efstratiou A et al. 2017). Moreover, the Center for Disease Control and Prevention (CDC) also reported that 32 outbreaks were caused by *Cryptosporidium* in the United States in 2016 mainly linked to activities in swimming pools and water playgrounds (https://www.cdc.gov/media/releases/2017/p0518-Cryptosporidium-outbreaks.html). At the same time, this parasite has also been reported to be responsible for more than 8 million cases of foodborne illness in 2010 and was ranked fifth out of 24 potential foodborne parasites (Ryan U et al. 2018). Moreover, the ECOPHIP team carried out the first epidemiological study to detect *Cryptosporidium* spp. in edible marine fish in European seas (Certad G et al. 2019). Additionally, novel *Cryptosporidium* fish genotypes were also identified (Certad G et al. 2020). I also participated in this study (Appendix 6.2; Article 1). Thus, indicating that edible marine fish pose a risk of zoonotic transmission of the parasite either through their consumption and/or

handling or through the consumption of water contaminated with fully sporulated oocysts shed in fish feces.

Even though, the health risk associated with the consumption of contaminated food is high, the techniques for detection of *Cryptosporidium* oocysts are generally inadequate, being laborious, time-consuming, and expensive (Chalmers RM et al. 2020). Recently, there has been some progress with attempts made to refine the existing protocol to improve the retrieval of the oocysts from challenging leafy green vegetables such as spinach leaves in a cost-effective way (Razakandrainibe R et al. 2020).

Following this brief update on the latest discoveries associated with the parasite in terms of its species, life cycle, transmission, and epidemiology, and before describing the different results obtained during my thesis project, I will present a state of the art about *Cryptosporidium* that I organized in three different chapters.

- ✓ The first chapter, "**Generalities**", gives an overview of what is known about *Cryptosporidium* and its impact on human health. In this chapter I integrated a review article entitled "***La cryptosporidiose et son impact en santé publique***", published in the "Revue de Biologie Médicale", Numéro 352 - Janvier-février 2020, that I wrote together with other laboratory members and colleagues in the field of Parasitology.

Briefly, this review includes aspects of the biology of the parasite, an update of its taxonomic characterization, a description of clinical manifestations which varies from mild to very severe depending upon the nutritional and immune status of the host, and an overview of various diagnostic tools currently under use to measure the ever-increasing epidemiological significance of the parasite. Finally, we emphasize the lack of available treatments and the need for further research to contribute to the understanding of the pathogenicity of this parasite.

- ✓ The second chapter, "**Epigenetics in Apicomplexan parasites**", provides an overview of the current advances in the understanding of the epigenetic mechanisms employed by different Apicomplexan parasites such as *Plasmodium falciparum*, *Toxoplasma gondii,* and *Theileria annulata*. This chapter highlights the important involvement of epigenetic mechanisms in the pathogenicity of the Apicomplexan parasites. In the case of *Cryptosporidium*, these aspects are still relatively unexplored.

- ✓ The third chapter, "***Cryptosporidium* and cancer**", includes a review article entitled "***Cryptosporidium* and colon cancer: cause or consequences?**", published in Microorganisms. 2020 Nov; 8(11): 1665, which was co-written with other members of the laboratory.

In this section, we provide an update on the latest literature available concerning epidemiological and experimental studies on this topic and give a critical opinion about possible mechanisms involved in this process of oncogenesis associated with the infection.

Finally, and to broaden my knowledge and skills in the field of parasitology, I had the opportunity during my thesis to be involved in three other research projects not developed in this report but listed in Appendix 6.2. The first one was about the epidemiology of *Cryptosporidium* in edible marine fish with the molecular identification of new genotypes (Appendix 6.2; Article 1). The second one focused on a major thematic of the team, which is the study of the prevalence and circulation of the intestinal protozoan *Blastocystis* in the human population through large epidemiological surveys carried out in Senegal (Appendix 6.2; Article 2), in Syrian refugee camps in North Lebanon (Appendix 6.2; Article 3) and more recently in Guinea (Appendix 6.2; Article 4) The last project considered as an expertise activity in the team was on the prevalence and molecular identification of protozoa belonging to the trichomonad group in wild wetland birds in the Netherlands in close collaboration with Royal GD, a leading Dutch organization in the field of animal health (Appendix 6.2; Article 5).

Introduction

1.1. Generalities

La cryptosporidiose et son impact en santé publique

M. Sawant [1], S. Benamrouz [1, 2], M. Chabé [1], K. Guyot [1], D. Costa [3], L. Favennec [3, 4], G. Gargala [3, 4], E. Viscogliosi [1], G. Certad [1, 5]

[1] CNRS, Inserm, CHU Lille, U1019 - UMR 8204 - CIIL - Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille, Université de Lille, Lille, France.

[2] Equipe Ecologie et biodiversité, Unité de Recherche Smart and Sustainable Cities, Faculté de Gestion Economie et Sciences, Institut Catholique de Lille, France

[3] Département de Parasitologie, Hôpital Universitaire de Rouen l, Rouen, France.

[4] Département de Médecine et de Pharmacie, Université de Normandie, Rouen, France.

[5] Délégation à la Recherche Clinique et à l'innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, Lille, France.

Résumé

Cryptosporidium est une cause majeure de diarrhée à travers le monde, et l'infection par ce parasite touche en particulier les jeunes enfants et les patients immunodéprimés. La sévérité de la maladie varie de légère à très grave et les signes et symptômes dépendent de l'état nutritionnel et immunitaire de l'hôte mais également des facteurs de virulence du parasite. Les sources et les voies de transmission impliquent à la fois une propagation zoonotique et une propagation anthroponotique. Cette transmission est facilitée par la résistance du parasite aux nombreux désinfectants couramment utilisés. Les mesures de prévention et de contrôle revêtent alors une importance majeure en vue de la protection des groupes vulnérables puisqu'en outre, les options de traitement sont limitées.

Mots clés :

Cryptosporidium, protozoose digestive, diversité génétique, épidémiologie moléculaire, transmission, pathogénicité, diagnostic, traitement, prévention

Introduction

***Cryptosporidium*, un agent pathogène?**

La cryptosporidiose est une maladie diarrhéique causée par *Cryptosporidium*, un parasite infectant principalement le tube digestif d'un nombre important de vertébrés, notamment l'Homme. Les parasites du genre *Cryptosporidium* sont des protistes intracellulaires appartenant au phylum des Apicomplexa.

Cryptosporidium a été décrit pour la première fois par Ernest Tyzzer en 1907. Tyzzer a isolé ce parasite à partir de la muqueuse gastrique de souris (*Mus musculus*) et l'a nommé *Cryptosporidium muris* (*C. muris*). En 1912, il décrit, également chez la souris, une autre espèce de plus petite taille, qu'il nomma C. *parvum*. Il s'agissait bien d'espèces différentes puisque cet auteur avait montré des différences morphologiques significatives des oocystes de ces deux isolats. De plus, *C. parvum* est localisé dans l'épithélium intestinal alors que C. *muris* est localisé au niveau des glandes gastriques (1).

C. meleagridis, une nouvelle espèce identifiée dans les intestins de dindons (*Meleagridis gallopavo*) a été décrite en 1955 et c'est dans ce cadre que pour la première fois, l'association entre le parasite et des manifestations cliniques a été évoquée. Cependant, *Cryptosporidium* sp. sera considéré comme un commensal jusque dans les années 70 où il sera reconnu comme responsable d'épidémies de diarrhées parfois mortelles dans les élevages de veaux (1).

Les premiers cas humains de cryptosporidiose ont été rapportés chez des patients souffrant de diarrhées aqueuses. Cependant, ce n'est qu'au début des années 80 que l'impact clinique de ce parasite a été reconnu, en raison d'une morbidité et d'une mortalité élevées chez les patients immunodéprimés et en particulier chez les patients atteints du SIDA (1). L'infection par *Cryptosporidium* sp. chez les individus VIH+ au stade SIDA s'avère chronique et peut même engager le pronostic vital du patient. Le lien de ce parasite avec le SIDA est même si fort que la cryptosporidiose est devenue l'un des critères définissant la maladie (OMS, 2007).

Grace à l'utilisation de la thérapie antirétrovirale, le nombre de cas de cryptosporidiose parmi les individus atteint du VIH/SIDA dans les pays développés a considérablement diminué. En reconstituant la fonction immunitaire, cette thérapie semble également réduire le taux d'infection parasitaire. Cependant, la cryptosporidiose demeure encore une complication grave du VIH/SIDA chez les patients sans accès à la multi-thérapie antirétrovirale, particulièrement dans les pays en voie de développement.

Cryptosporidium sp. est également considéré comme l'agent infectieux responsable de plus de 60% des épidémies d'origine hydrique (2). Les épidémies de cryptosporidiose touchent les pays développés comme les pays en voie de développement, en zones aussi bien urbaines que rurales (3). De nombreuses épidémies ont été rapportées entre 1984 et 1999, principalement en

Amérique du Nord, au Royaume-Uni et au Japon. C'est toutefois en 1993 qu'une soudaine épidémie touche plus de 400 000 personnes à Milwaukee (Winsconsin, Etats-Unis) à la suite d'une contamination du réseau de distribution d'eau. Près de 4000 hospitalisations furent répertoriées ainsi que 69 décès (4). Au Royaume-Uni et au Pays de Galles, *Cryptosporidium* sp. a été impliqué dans 70% des épidémies de gastro-entérite infectieuse entre 1992 et 2003 (5). En France aussi, des épidémies de cryptosporidiose liées à la contamination de l'eau de distribution par *Cryptosporidium* sp. ont été décrites (6).

Des épidémies de cryptosporidiose d'origine alimentaire ont également été rapportées dans différentes régions du globe liées à la consommation d'aliments aussi divers que les jus de pommes, le lait, les oignons crus ou la salade. Plus spécifiquement en Europe, *Cryptosporidium* sp. a été classé en 5ème position des parasites d'origine alimentaire à prendre en compte de façon prioritaire (7).

L'oocyste qui est la forme du parasite responsable de la transmission possède une paroi épaisse ce qui explique sa survie prolongée dans l'environnement et sa résistance aux produits de désinfection usuels, dont l'eau de Javel (chlore). La résistance des oocystes de *Cryptosporidium* sp. dans l'eau et dans l'environnement, l'absence de traitement ou de vaccination chez les humains et les animaux, ainsi que ses implications socio-économiques, ont conduit l'Organisation Mondiale de la Santé (OMS) à inclure la cryptosporidiose dans la liste des maladies négligées (8).

Depuis l'émergence de *Cryptosporidium* sp. liée à la pandémie de SIDA, suite à l'épidémie de Milwaukee, et sous la forte impulsion des industries de l'eau, les études autour de ce parasite se sont multipliées afin de mieux comprendre sa biologie, de développer des méthodes de détection, de définir une stratégie de prévention, et de mettre au point un traitement efficace.

En effet, la cryptosporidiose est également une pathologie fréquente chez les jeunes enfants et récemment, les résultats du Global Enteric Multicenter Study (GEMS) ont montré que

Cryptosporidium spp. était l'une des principales causes de diarrhées modérées à sévères et la 2ème cause de mortalité par diarrhée après rotavirus chez les enfants de moins de 2 ans (9,10).

Cycle biologique

Cryptosporidium est un parasite protozoaire intracellulaire qui infecte l'épithélium gastro-intestinal de nombreux vertébrés. Son cycle de vie est monoxène et débute par l'ingestion d'oocystes sporulés, très résistants dans l'environnement et contenant 4 sporozoïtes (Figure 1). Une fois dans le tractus gastro-intestinal, ces oocystes subissent une désenkystement qui permet la libération des sporozoïtes. Plusieurs facteurs semblent favoriser ce phénomène tels que la

température, le pH, le CO_2, les enzymes pancréatiques et les sels biliaires. Certains facteurs dérivés des parasites comme la sérine, la protéase cystine, l'arginine aminopeptidase, la phospholipase sécrétoire A2 et les molécules associées à la synthèse des protéines, y compris les protéines associées aux ribosomes et aux chocs thermiques sont impliquées dans le déclenchement du processus de désenkystement. Une fois les sporozoïtes libérés dans la lumière intestinale, ceux-ci vont pénétrer les cellules épithéliales (11).

Leur premier contact se fait tout d'abord avec la couche muqueuse supérieure des cellules épithéliales. Cependant, le mécanisme par lequel le parasite franchit cette barrière n'est pas encore clarifié. Cela pourrait être dû à des protéases secrétées par *Cryptosporidium* ou tout simplement à un phénomène mécanique ou encore à un tout autre mécanisme inconnu pour le moment. Toutefois, son attachement aux cellules épithéliales est médié par une variété de molécules exprimées à la surface du sporozoïte qui peuvent se lier au récepteur de la cellule hôte ou aux structures de surface (11).

Les sporozoïtes sont ensuite intériorisés dans une structure bimembranaire formant ce qui est appelé la vacuole parasitophore. Cette structure a une localisation unique au niveau apical de la cellule hôte et est considérée comme intra-membranaire mais extra-cytoplasmique. À l'intérieur de la vacuole parasitophore, le sporozoïte se développe en trophozoïte. Ce dernier entre dans une reproduction asexuée pour former un méronte de type I. La division cellulaire de ce dernier entraîne la formation de cellules filles, appelées merozoïtes, chacun entouré de sa propre membrane. Les mérontes de type I libèrent ainsi 8 mérozoïtes, qui envahissent ensuite les cellules épithéliales voisines. Les mérozoïtes de type I peuvent aussi se développer en un autre méronte de type I en subissant une division asexuée (mérogonie) ou peuvent continuer à se développer pour former des mérontes de type II. Les mérontes de type II produisent quatre mérozoïtes qui se fixent à nouveau aux cellules épithéliales. Par des mécanismes encore inconnus, ces mérozoïtes sont programmés pour se différencier en microgamétocytes ou en macrogamétocytes. Jusqu'à seize microgamètes peuvent être identifiés dans un microgamétocyte. Ces microgamètes finissent par féconder un macrogamétocyte pour former un zygote diploïde qui subit un processus similaire à la méiose (sporogonie), donnant ainsi naissance à quatre sporozoïtes haploïdes dans un oocyste (oocyste sporulé). Les oocystes résultants sont à paroi fine ou épaisse et sont libérés dans la lumière de l'intestin puis sécrétés dans l'environnement pour infecter d'autres hôtes (12).

Introduction

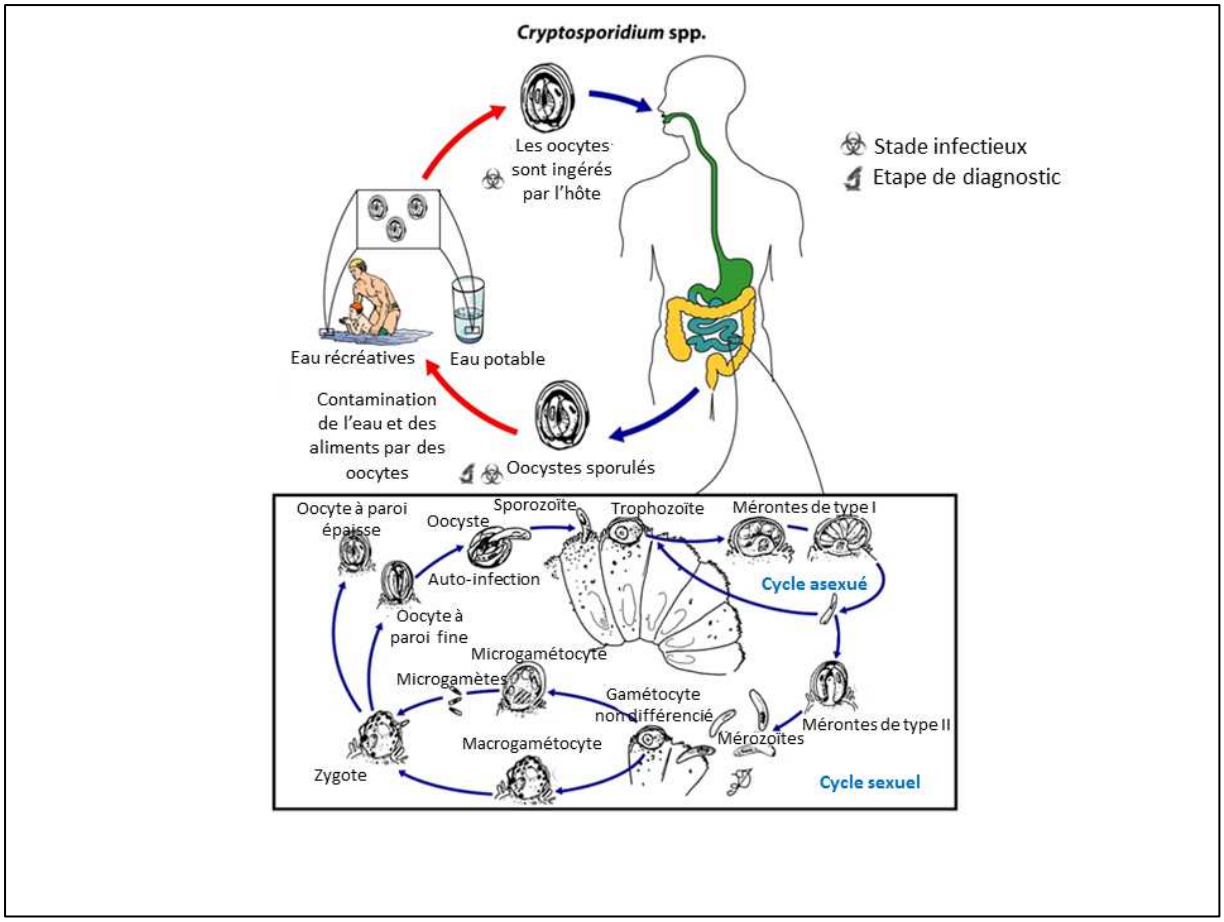


Figure 1. Cycle de vie de Cryptosporidium (Source ep Centers for Disease Control and Prevention: https://www.cdc.gov/dpdx/cryptosporidiosis/index.html).

Taxonomie de *Cryptosporidium*

Une coccidie atypique ou une grégarine ?

Depuis sa découverte *Cryptosporidium* a été considéré comme une coccidie appartenant au phylum Apicomplexa. Cependant, de nombreuses caractéristiques du parasite ont mis en doute son appartenance au groupe des Coccidies et ont conduit certains auteurs à le considérer comme une coccidie atypique plus proche des Grégarines. Quelques caractéristiques de *Cryptosporidium* qui permettent de le différencier des coccidies traditionnelles sont les suivantes: (i) sa forme infectante est l'oocyste sporulé permettant l'auto-infection, ce qui est une stratégie de développement plus complexe, (ii) *Cryptosporidium* possède un organelle d'attachement, (iii) l'apicoplaste est absent, (iv) sa localisation est extracytoplasmique, et (v) le parasite est insensible aux traitements anti-coccidies (13).

De plus, une analyze phylogénétique montrait déjà en 1999, que le clade composé par les Grégarines et *Cryptosporidium* était séparé des autres Apicomplexa y compris des Coccidies (14). Ceci a été confirmé depuis par d'autres équipes qui ont réussi à mettre en évidence en

cultures cellulaires ou axéniques, des formes extracellulaires du parasite (15). Cela signifie que le parasite peut achever un cycle en absence de la cellule hôte d'où le fait de considérer ce parasite comme une « Grégarine-like » (15). De nos jours, les preuves en faveur de cette hypothèse ne cessent de s'accumuler. Certains auteurs apportent des arguments structuraux en décrivant la capacité du parasite à modifier sa structure en fonction de son environnement (16). Grâce notamment à l'utilisation de la microscopie électronique à transmission il a été rapporté la présence d'un stade extracellulaire de gamontes-like *in vitro* dans les cultures cellulaires et axéniques, qui serait une forme de trophozoïtes n'ayant pas réussi à pénétrer dans la cellule (16). A la lumière de l'ensemble des descriptions de stades extracellulaires du parasite, une représentation schématique de la formation du feeder-organelle (épimerite) dans des conditions épicellulaires et extra-cellulaires a été proposée (17). D'autres auteurs précisent ou corrigent ce que nous savions de la biologie et du cycle de vie du parasite en soulignant que celui-ci peut également se multiplier sans l'intervention de la cellule épithéliale dans la nature en milieu aquatique sur des biofilms (15,18). Plus encore, il a été suggéré que même sur des cultures cellulaires HCT8 infectées par *C. parvum*, le parasite semble, au fil du temps, être majoritairement représenté par des formes extracellulaires de trophozoïtes et de mérontes non attachées à la cellule hôte. Il semblerait donc que les deux cycles de vie du parasite, intra et extracellulaire, soient co-existants (19). Enfin, l'évolution des outils de biologie moléculaire et de phylogénie a permis d'apporter de nouveaux éléments. En effet, Cavalier-Smith, grâce à une analyze phylogénétique des sites hétérogènes de l'ADNr 18S de 122 grégarines et 452 groupes extérieurs, a permis de proposer une nouvelle classification des Grégarines (20). Dans celle-ci, *Cryptosporidium* fait partie de la sous-classe des Cryptogregaria (dont il est le seul représentant à ce jour) au sein de la classe des Gregarinomorphea (20). Cette sous-classe serait composée de parasites épicellulaires infectant les vertébrés, possédant un feeder-organelle mais n'ayant plus d'apicoplaste (17). A l'heure actuelle, une seule publication parue en 2016 a considéré que *Cryptosporidium* était officiellement une Grégarine (16), ce qui en ferait le seul parasite appartenant à ce groupe, capable d'infecter des vertébrés.

Les espèces de Cryptosporidium

A ce jour, 43 espèces (Tableau 1) et génotypes de *Cryptosporidium* ont été identifiés chez différents groupes d'animaux comme les mammifères, les oiseaux, les reptiles, les amphibiens et les poissons. Parmi ces espèces, seules 31 d'entre elles sont considérées comme valides à l'heure actuelle (21–23).

Introduction

Ce n'est pas encore le cas de *C. agni, C. nascoris, C. ducismarci* et *C. pestis*, le plus souvent par manque voir même l'absence de données morphologiques, biologiques ou taxonomiques suffisantes, ne leur permettant pas de répondre aux règles définies par l'ICZN (International Code of Zoological Nomenclature) (24). Dans le cas plus spécifique de *C. tyzzeri*, certains auteurs avaient suggéré que ce taxon était un « junior synonym » de *C. meleagridis* (22). Cependant, les analyzes moléculaires ont permis de les identifier comme deux espèces indépendantes.

Vingt espèces (Tableau I) et génotypes de *Cryptosporidium* sp. ont été rapportés comme pouvant infecter l'homme incluant *C. parvum*, *C. hominis*, *C. meleagridis, C. felis, C. canis, C. ubiquitum, C. cuniculus, C. viatorum, C. erinacei, C. fayeri, C. scrofarum, C. tyzzeri*, *C. andersoni, C. suis, C. bovis, C. xiaoi,* un génotype de cheval, un génotype de mouffette, un génotype de vison et le génotype chipmunk. Il est important de noter que *C. hominis* et *C. parvum* sont à eux seuls, responsables de plus de 90% des cas de cryptosporidiose humaine (24). Il a également été rapporté très récemment que *C. parvum* pouvait être subdivisé en deux sous espèces, *C. parvum anthroponosum* (*C.p.anthroponosum*), qui serait anthroponotique puisqu'elle infecterait presque exclusivement l'Homme, et *C. parvum parvum* (*C.p.parvum*) qui serait plutôt zoonotique infectant également l'Homme mais principalement les animaux (25).

Tableau I. Espèces de *Cryptosporidium* et leur association avec la cryptosporidiose humaine

Espèce de *Cryptosporidium*	Hôte (s) principal (s)	Présence chez l'homme	Références
C. muris	Rongeurs	Oui	Tyzzer (1907)
C. parvum	Humains ; mammifères	Oui	Tyzzer (1912)
C. meleagridis	Oiseaux	Oui	Slavin (1955)
C. wrairi	Cochon d'inde	Non	Vetterling et al. (1971)
C. bovis	Bovins	Oui	Barker & Carbonell (1974)
C. agni	Moutons	Non	Baker & Carbonell (1974)
C. cuniculus	Lapins	Oui	Inman & Takeuchi (1979)
C. felis	Chats	Oui	Iskeki (1979)
C. serpentis	Reptiles	Non	Levine (1980)
C. nascoris	Poissons	Non	Hoover (1981)
C. baileyi	Poulet	Non	Upton& Haynes (1986)
C. varanii	Reptiles	Non	Palvàsek (1995)

C. galli	Poulet	Non	Palvàsek (1999)
C. andersoni	Bovins	Oui	Lindsay (2000)
C. canis	Chiens	Oui	Fayer (2001)
C. hominis	Humains	Oui	Morgan-Ryan (2002)
C. molnari	Poisson	Non	Sijta-Bobadilla (2002)
C. suis	Porc	Oui	Ryan (2004)
C. scophthalmi	Poisson	Non	Alvarez Pellitero (2004)
C. pestis	Mammifères	Oui	Šlapeta (2006)
C. fayeri	Marsupial	Oui	Ryan (2008)
C. ryanae	Bovins	Non	Fayer (2008)
C. fragile	Crapaud épineux-noir	Non	Jirku (2008)
C. macropodum	Kangourou gris de Pâques	Non	Power & Ryan (2008)
C. ducismarci	Reptiles	Non	Traversa (2010)
C. xiaoi	Moutons	Non	Fayer (2009)
C. ubiquitum	Mammifères	Oui	Fayer (2010)
C. tyzzeri	Souris	Oui	Zhao (2012) Slapeta et al. (2012)
C. viatorum	Humains	Oui	Elwin (2012)
C. scrofarum	Porc	Oui	Kvác (2013)
C. erinacei	Mammifères	Non	Kvác (2014)
C. huwi	Poisson	Non	Ryan (2015)
C. rubeyi	Écureuil	Non	Xunde Li (2015)
C. proliferans	Mammifères	Non	Kvác (2016)
C. avium	Oiseaux	Non	Hulubová (2016)
C. testudinis	Reptiles	Non	Ježková (2016)
C. homai	Porc	Non	Zhahedi (2017)
C. alticolis	Campagnols communs	Non	Horčičková (2018)
C. microti	Campagnols communs	Non	Horčičková (2018)
C. occulutus	Rats	Non	Kvác (2018)
C. apodemi	Apodèmes	Non	Čondlová (2018)
C. ditrichi	Apodèmes	Non	Čondlová (2018)
C. proventriculi	Avian	Non	Holubová (2019)

Données compilées à partir des travaux de (22) et (24)

Cryptosporidium à l'ère post-génomique

Les génomes des isolats de *C. parvum* IOWA II, *C. hominis* (TU502) et *C. muris* (RN66) (disponibles dans la base de données CryptoDB ; http://cryptodb.org) ont été séquencés il y a plus d'une décennie. Plus récemment, d'autres isolats de *C. parvum*, *C. hominis* et d'autres espèces ou génotypes du genre *Cryptosporidium* telles que *C. baileyi*, *C. meleagridis*, *C. andersoni*, *C. tyzzeri*, *C. ubiquitum* et *Cryptosporidium* chipmunk genotype I ont également vu

leurs génomes séquencés, ceux-ci étant également disponibles dans CryptoDB. De façon générale, les espèces de *Cryptosporidium* possèdent un génome assez compact par rapport à celui d'autres parasites phylogénétiquement proches tels que *Plasmodium falciparum* et *Toxoplasma gondii*. Cette taille réduite est en partie due à l'absence d'apicoplaste chez cet Apicomplexa particulier ainsi qu'à un métabolisme rationalisé dépendant en grande partie de l'acquisition de nutriments via son hôte (26).

Cette collection de données génomiques ouvre aujourd'hui le champ à la post-génomique. Pour *C. parvum*, la mise en œuvre d'études transcriptomiques et protéomiques à grande échelle s'est avérée très difficile, principalement en raison de la difficulté d'isoler les stades intracellulaires de multiplication du parasite. Cependant, les progrès récents dans le développement de nouveaux modèles *in vitro* sont très prometteurs dans ce domaine de recherche. A notre connaissance, outre des travaux réalisés en utilisant des techniques de RT-qPCR (reverse-transcriptase quantitative-PCR) ou de puces à ADN, seules trois études de RNA-seq (séquençage aléatoire du transcriptome entier) ont été menées chez *C. parvum*. Il s'agit : (i) des travaux de Lippuner *et al*. en 2018 qui ont étudié l'expression des gènes de *C. parvum* dans les sporozoïtes et les stades intracellulaires du parasite en culture ou dans l'épithélium intestinal de veaux infectés par *C. parvum*, (ii) de Heo *et al.* en 2018 sur des organoïdes épithéliaux pulmonaires ou intestinaux humains infectés par le parasite, et (iii) ceux de Matos *et al*. en 2019 sur des oocystes, sporozoïtes et des cellules épithéliales MDBK infectées par *C. parvum* à 2, 24 et 48 heures après infection (27,28). Dans ce dernier travail (28), les auteurs mettent en évidence des différences significatives entre les transcriptomes exprimés à l'extérieur et à l'intérieur de la cellule hôte. Les données transcriptomiques des stades parasitaires intracellulaires indiquent que le transcriptome intracellulaire de *Cryptosporidium* est configuré pour des processus biologiques tels que la transcription et la traduction, certainement en relation avec une multiplication asexuée rapide pendant la phase initiale de l'infection. De plus, les gènes significativement surexprimés dans les oocystes sont associés à des fonctions non retrouvées chez d'autres Apicomplexa.

Par ailleurs, il a également été suggéré que *Cryptosporidium* est capable de modifier l'expression génétique des cellules hôtes (29). Ainsi, les profils de transcription des gènes suggèrent que l'expression des gènes pro-apoptotiques de l'hôte est régulée négativement au début de l'infection par le parasite, mais qu'elle est favorisée à un stade plus avancé du cycle parasitaire. De plus, des travaux intéressants récemment publiés ont montré que certains transcripts de *C. parvum* peuvent être délivrés sélectivement dans les noyaux des cellules

épithéliales de l'hôte pendant l'infection et peuvent moduler la transcription des gènes dans les cellules infectées de l'hôte (30).

Pouvoir pathogène du parasite

La pathophysiologie des diarrhées causées par *Cryptosporidium* sp. est multifactorielle et inclut des facteurs de l'hôte ainsi que des facteurs parasitaires. Cependant, les mécanismes exacts contribuant à la maladie causée par *Cryptosporidium* sp. ne sont pas totalement élucidés.

Concernant le fort pouvoir infectieux de *Cryptosporidium* sp., celui-ci a été mis en évidence à la suite de nombreuses études ayant établi que de très faibles doses du parasite peuvent induire une infection chez l'Homme et certains modèles murins. Chez les patients immunocompétents naïfs vis-à-vis de la cryptosporidiose, la dose infectante est de l'ordre de 10 à 1000 oocystes selon les isolats (31). La dose infectante chez les patients ayant déjà contracté une infection est environ 30 fois plus élevées (32). Ceci explique que la cryptosporidiose se manifeste cliniquement principalement chez les enfants. Cependant il faut préciser que l'infectivité (ID50 : dose infectieuse pour 50% de la population) du parasite chez l'Homme dépend aussi des isolats parasitaires et de leur origine géographique.

La capacité immunitaire de l'hôte est un déterminant critique de la probabilité et de la gravité de l'infection par *Cryptosporidium* sp.. Auto-résolutive chez les sujets immunocompétents, la maladie devient chronique voire létale chez les personnes immunodéprimées (33). Parmi les étiologies d'immunodépression impactant la sévérité de la cryptosporidiose, on retrouve principalement les troubles immunosuppresseurs affectant la fonction des lymphocytes T tels que le VIH (principalement les patients VIH+ avec un taux de CD4 < 50/mm^3) et des déficiences immunitaires primaires telles que les déficits immunitaires combinés sévères ou le syndrome hyper-IgM lié à l'X.

La multiplication de *Cryptosporidium* sp. dans les entérocytes, au sein de sa vacuole parasitophore, entraîne des perturbations hydroélectriques et une malabsorption intestinale dont le résultat final est une diarrhée. Plusieurs mécanismes physiopathologiques de cette diarrhée induite par le parasite ont été proposés: (i) l'infiltration de la *lamina propria* par les cellules immunitaires de l'hôte (lymphocytes, macrophages et neutrophiles), responsable de diarrhée inflammatoire, (ii) une perméabilité trans-épithéliale accrue, avec rupture des jonctions inter-cellulaires, atrophie villositaire, hyperplasie des cryptes et mort cellulaire, caractéristiques d'une diarrhée exsudative et (iii) une malabsorption due à la perte de l'architecture intestinale, liée à une diarrhée osmotique (34).

Introduction

Du fait de l'absence d'un modèle de culture continue du parasite et de l'incapacité, jusqu'à récemment, de le transfecter (35,36), l'étude des facteurs de virulence de *Cryptosporidium* sp. restent à ce jour parcellaire. Cependant, des méthodes immunologiques et moléculaires ont permis de mettre en évidence certains candidats tels que des protéines impliquées dans le désenkystement des oocystes, la motilité du parasite, son adhérence à la cellule hôte (comme les glycoprotéines de type mucine et les protéines adhésives apparentées à la thrombospondine), l'invasion des cellules épithéliales par les sporozoïtes, la formation de la vacuole parasitophore, la multiplication intra-cellulaire et les dommages causés aux cellules

hôtes (37). Plusieurs molécules, telles que des phospholipases, des protéases et l'hémolysine H4 ont été proposées comme la cause de ces dommages cellulaires (37). Par ailleurs, *Cryptosporidium* sp. est également capable de moduler l'apoptose des cellules hôtes. Ainsi, le parasite inhibe l'apoptose cellulaire au stade trophozoïte, mais favorise ce processus aux stades sporozoïte et mérozoïte (33).

Epidémiologie de la cryptosporidiose

La cryptosporidiose est récemment apparue comme l'une des principales causes de diarrhées parasitaires chez l'Homme à travers le monde. Du fait de la contamination de l'environnement, il faut différencier l'épidémiologie de la cryptosporidiose dans les pays en voie de développement de celle des pays développés.

Dans les pays en voie de développement, la cryptosporidiose est apparue comme la deuxième cause de mortalité par diarrhée chez les enfants âgés de 12 à 24 mois en Afrique et en Inde (9) (10). Dans ces pays, la transmission de la maladie due à *C. parvum* et *C. hominis*, est principalement interhumaine (38). Les facteurs de risque retrouvés sont une densité élevée de population, des modes de vie avec des conditions d'hygiène très précaires, une absence de traitement des eaux usées, et le jeune âge. On estime ainsi le nombre de cas de cryptosporidiose dans les pays en voie de développement à 7,6 millions par an.

Dans les pays développés et en particulier en Europe occidentale, cette infection reste sous diagnostiquée et son importance méconnue. Dans les pays où des études ont été réalisées dans la population générale, la prévalence de la cryptosporidiose varie entre 770 cas pour 100 000 habitants en Angleterre à 4000 cas pour 100 000 habitants aux Pays-bas. On estime que parmi ces patients, environ 1/10 consultera un médecin généraliste et 2/100 auront un examen des selles pour la recherche de *Cryptosporidium* sp. (39). En France, dans les laboratoires d'analyze qui recherchent systématiquement ce parasite lors d'une demande d'examen parasitologique

des selles, environ 1,2 % des selles analysées sont positives. Toutefois dans la grande majorité des cas, cette parasitose en France reste méconnue chez les médecins généralistes et n'est pas diagnostiquée.

Dans la population immunocompétente française, si la majorité des cas de cryptosporidiose touche les enfants de moins de cinq ans, le nombre de cas apparaissant chez des adolescents voire chez des jeunes adultes est de plus en plus important. Ceci pourrait être dû à l'amélioration des conditions d'hygiène et en particulier à l'amélioration du traitement des eaux destinées à la consommation humaine aboutissant à un premier contact plus tardif avec ce parasite.

Les principaux facteurs de risque identifiés en France pour la cryptosporidiose sont 1) un contact avec un animal et en particulier un animal de rente, 2) une baignade en eau douce, que ce soit en eau vive ou en piscine, et 3) un contact avec un individu infecté (changement de couches d'un enfant malade, par exemple). Il existe en France comme dans le reste de l'Europe une forte saisonnalité pour cette infection puisque la grande majorité des cas apparait en été et au début de l'automne (rapport Centre National de Référence Laboratoire Expert (CNR) Cryptosporidioses, 2018). Ceci pourrait être dû aux habitudes alimentaires ou aux modes de vie modifiés durant les périodes de congés.

D'un point de vue parasitaire, si en Europe le nombre de cas de cryptosporidiose dus à *C. parvum* est à peu près équivalent à ceux dus à *C. hominis,* cette dernière espèce est très minoritaire en France puisqu'elle représente moins de 20 % des cas. D'un point de vue moléculaire, la majorité des cas de cryptosporidiose à *C. parvum* sont dus en France comme dans le reste du monde au sous-type IIaA15G2R1 et pour *C. hominis* au sous-type IbA10G2.

Les vecteurs environnementaux de *Cryptosporidium* sp. pouvant être à l'origine d'infections sont naturellement l'eau du robinet mais également des végétaux contaminés consommés crus comme des salades ou des condiments (basilique, coriandre).

En dehors des cas sporadiques, des épidémies françaises ont pu être identifiées depuis la mise en place en 2017 du CNR Cryptosporidioses. Ainsi une épidémie de plus de 150 personnes liée à la contamination de l'eau du robinet a été détectée dans le Lot-et-Garonne à l'été 2017 et deux autres épidémies d'origine hydrique ont été détectées en Guyane en 2017 puis en 2018. La cryptosporidiose peut-être également due à la contamination de produits laitiers puisqu'une épidémie est survenue dans un collège de Loire-Atlantique associée à la consommation de fromage blanc au lait cru (rapport CNR Cryptosporidioses, 2018). La cryptosporidiose apparaît donc de plus en plus comme une infection pouvant être d'origine alimentaire.

Introduction

Chez l'immunodéprimé, et du fait des conséquences cliniques plus évidentes de l'infection qui n'est pas spontanément résolutive, la cryptosporidiose est plus facilment diagnostiquée. En France, environ une centaine de cas de cryptosporidiose chez les patients immunodéprimés est détectée chaque année (40). Cela concerne pour la moitié des cas des patients ayant bénéficié d'une transplantation d'organe solide, majoritairement des patients transplantés de rein. Un quart des patients immunodéprimés et infectés par *Cryptosporidium* sp. sont des patients infectés par le VIH, l'autre quart étant constitué de patients souffrant des autres types d'immunodépressions (maladies auto-immunes, déficits immunitaires...). En France, le taux de létalité chez les immunodéprimés est de l'ordre de 5%.

Présentation clinique de la cryptosporidiose

Les manifestations cliniques et la sévérité de la cryptosporidiose peuvent varier d'une personne à l'autre en fonction de l'état immunitaire de l'individu allant de la présentation asymptomatique jusqu'à la maladie grave (33). Le symptôme principal le plus rapporté reste la diarrhée. D'autres symptômes tels que les crampes abdominales, les nausées, les vomissements, la perte de poids, la fièvre, l'anorexie et la fatigue ont également été signalés. Chez les individus immunocompétents, la durée des symptômes est en moyenne de 12 jours et ces manifestations cliniques sont spontanément résolutives (41). Chez les patients immunocompromis par différentes causes telles que la malnutrition, l'infection par le VIH ou le cancer, une diminution des lymphocytes T CD4+ est associée à un risque accru de développer une cryptosporidiose (42).

Chez ces derniers, l'infection peut devenir chronique tant que l'immunodépression persiste. La cryptosporidiose peut être létale du fait des diarrhées fréquentes et volumineuses conduisant à une déshydratation très grave du patient. Des sites extra-intestinaux tels que les voies biliaires, les poumons et le pancréas peuvent être infectés chez les patients quel que soit leur statut immunitaire. L'infection des voies biliaires peut entraîner une cholangiopathie caractérisée par des symptômes comme la fièvre, la douleur abdominale, des nausées, des vomissements, de la diarrhée et de l'ictère. Une élévation de la bilirubine sérique et des enzymes hépatiques peut également y être associée (42). Les symptômes de la cryptosporidiose respiratoire peuvent inclurent toux sèche et dyspnée productive (43).

Certains rapports ont décrit différents types de présentations de la cryptosporidiose chez les patients atteint par le SIDA tels que 1) les infections asymptomatiques, 2) les infections transitoires chez les sujets moins immunodéprimés, 3) la maladie fulminante caractérisée par

plus de 2 L de selles/jour chez les patients dont la numération des lymphocyte CD4+ est inférieure à 50/mm^3, 4) l'infection chronique (43).

Des récidives de troubles gastro-intestinaux mais également de manifestations non gastro-intestinales à long terme ont été rapportées en tant que séquelles cliniques de la cryptosporidiose chez les patients immunocompétents (44).

Manifestations cliniques chez l'homme en fonction des espèces

Des différences dans les manifestations cliniques ont été observées entre différentes espèces de *Cryptosporidium*, en particulier entre *C. hominis* et *C. parvum*, le premier étant plus virulent (33). Il a été signalé que *C. hominis* est plus souvent associé à des douleurs oculaire et articulaire, céphalée, et fatigue que *C. parvum* (44).

Des associations ont également été observées entre différents génotypes et sous-génotypes de *Cryptosporidium* par rapport aux manifestations cliniques (33). Par exemple, les infections par *C. hominis* sous-type Id, *C. parvum*, *C. canis* ou *C. felis* peuvent être plus sévères chez les personnes atteintes par le VIH+/SIDA. Dans ces cas, la diarrhée chronique peut se compliquer par un *wasting syndrome*. Il a été également montré que des infections par *C. meleagridis* peuvent être asymptomatiques et que les personnes atteintes par cette espèce excrètent en général moins d'oocystes que celles infectées par d'autres espèces de *Cryptosporidium* En outre, chez les patients infectés par *C. hominis* sous-type Id, le risque de diarrhée est plus élevé que chez les personnes infectées par le sous-type Ib. Le sous-type Ia de *C. hominis* n'a quant à lui pas été associé à la diarrhée dans cette étude (45).

Des études cliniques chez des volontaires sains ont montré également que différentes souches de *C. parvum* (UCP, Iowa, TAMU) avaient des ID50s (dose infectieuse 50%) différents, indiquant des variations dans leur pouvoir infectieux (33).

***Cryptosporidium* et cancer : cause ou conséquence ?**

Depuis plus d'un siècle, le nombre de travaux rapportant les preuves d'un lien entre les infections et le cancer n'ont cessé de s'accumuler (46). Ainsi, on estime que 25 à 50% des cancers humains seraient dues à des agents infectieux. En France par exemple, sur les 352 000 nouveaux cas de cancer recensés en 2015, 4.1% ont été attribués à des agents infectieux (47). Il s'agirait le plus souvent de virus (Epstein-Barr virus (EBV), Virus de l'hépatite B et C, papilloma virus humain…), de bactéries (*Helicobacter pylori*) ou de parasites.

Introduction

Sans que cela soit reconnu officiellement par l'International Agency for Research on Cancer (IARC), le cas de *Cryptosporidium* est particulièrement intéressant dans la mesure où la preuve de son lien avec les cancers gastro-intestinaux a été rapportée non seulement par des études de cas et des études épidémiologiques mais également expérimentalement aussi bien en modèle *in vivo* qu'en modèle *in vitro*.

En effet, *Cryptosporidium* en tant qu'agent opportuniste, est connu comme pouvant infecter de manière plus importante les patients immunodéprimés y compris ceux atteints de cancer. Un nombre croissant d'études épidémiologiques ont mis en évidence une prévalence significativement plus élevée de *Cryptosporidium* chez les individus atteints de cancer comparativement aux autres populations témoins. Ces travaux ont été réalisés sur des populations d'âges et de statut immunitaire différents et sur des patients atteints de différents types de cancer (48). Cependant, il a également été suggéré que *Cryptosporidium* peut être une cause possible de développement d'un cancer digestif chez l'Homme.

En effet, l'un des premiers cas liant le développement d'un adénocarcinome colique à la présence de *Cryptosporidium* date de la fin des années 80 en Espagne. S'en est alors suivie une série d'autres rapports associant la présence du parasite avec des lésions néoplasiques notamment au niveau du pancréas et des voies biliaires (49).

Plus récemment, deux études ont notamment été réalisées en Pologne en 2007 et 2012 sur des patients atteints de cancers colorectaux qui ont montré des prévalences respectivement de 18 et 12,6% de *Cryptosporidium* dans les selles de ces individus. Ce travail a été complété par ce même groupe via une autre étude réalisée en 2018 dans laquelle les auteurs ont montré une prévalence du parasite de 13% chez des patients atteints de cancers colorectaux comparativement à celle du groupe population contrôle sans cancer colorectal, qui n'était que de 4% (50). De plus, une étude réalisée au Liban a permis d'analyzer la prévalence du parasite chez des populations de patients auxquels un cancer colorectal avait été diagnostiqué avant tout traitement. Deux autres groupes contrôles ont également été étudiés, l'un constitué de patients symptomatiques ne présentant pas de lésions cancéreuses et l'autre présentant un cancer gastrique. L'identification du parasite a été réalisée sur les tissus et non dans les selles et a permis de mettre en évidence une prévalence de 21% chez les patients atteints de cancer colique. Cette valeur était significativement plus élevée que celle trouvée dans les deux populations témoins qui était respectivement de 6 et 0%. Ce travail a permis d'associer l'infection par l'espèce *C. parvum* à la pathologie cancéreuse mais également et pour la

première fois, l'espèce *C. hominis* (51). Ceci a été confirmé par une autre étude réalisée en Tunisie sur différents groupes de patients, qui montre une prévalence du parasite de près de 30% chez les patients atteints de cancer colorectal. Le génotypage réalisé lors de cette étude met en évidence la présence des deux espèces *C. parvum* et *C. hominis* (52). La présence de *C. meleagridis* a également été rapportée très récemment dans les biopsies d'un patient atteint de cancer colique (53). A l'heure actuelle, même appuyée par ces études épidémiologiques, le rôle que joue *Cryptosporidium* dans l'induction de tumeurs malignes gastro-intestinales chez l'Homme demeure incertaine au regard de son caractère opportuniste.

L'hypothèse de son lien avec l'induction du cancer a également été étayée par des preuves expérimentales. En effet, des souris SCID (Severe Combined Immunodeficiency) infectées par *C. parvum* développent, dès 45 jours PI des néoplasies intraépithéliales et des adénocarcinomes gastro-intestinaux et ce même avec de très faibles inocula (49). Ces lésions sont induites par toutes les souches de *C. parvum* testées et évoluent vers des adénocarcinomes invasifs atteignant les couches les plus externes des tissus (49). De plus, Baydoun et al ont mis au point un système de culture 3D d'explants entériques qui a non seulement permis de cultiver *C. parvum* pendant au moins 27 jours mais aussi de mettre en évidence déjà à ce stade de l'infection, la présence de lésions neoplasiques de bas grade par analyze histologique des tissus (54). Ce travail représente la première tentative réussie de développement *in vitro* d'une néoplasie colique induite par un parasite.

De manière générale, les infections peuvent conduire au développement de cancer par diverses voies. Certains agents agissent directement sur les gènes pour induire une prolifération cellulaire, d'autres causent des inflammations chroniques et d'autres enfin agiraient sur le système immunitaire en l'empêchant de détruire les cellules tumorales. Concernant *Cryptosporidium* sp. les mécanismes par lesquels il induirait le cancer ne sont pas encore élucidés. Cependant, des travaux récents montrent que des altérations de la voie de signalisation Wnt et du cytosquelette semblent être des événements majeurs lors du développement des lésions néoplasiques chez la souris (55). D'autres études sont nécessaires pour mieux comprendre le processus de cancérogénèse induit par *C. parvum*.

Diagnostic de la cryptosporidiose

Les symptômes de la cryptosporidiose ne sont pas assez spécifiques pour permettre de poser un diagnostic clinique différentiel. Le diagnostic différentiel de routine porte habituellement sur d'autres causes de gastro-entérites infectieuses telles que *Giardia, Cyclospora,*

Introduction

Cystoisospora, les microsporidies, les norovirus et rotavirus, *Campylobacter, Salmonella, Shigella* et *Escherichia coli*. Il est donc nécessaire d'utiliser différentes techniques de diagnostic parasitologiques ou biologiques.

Le diagnostic précis de l'infection par *Cryptosporidium* sp. est essentiel pour le contrôle de cette maladie et pour la compréhension de son épidémiologie. Différentes techniques utilisées pour détecter la présence du parasite chez l'Homme, chez l'animal ou dans des échantillons environnementaux sont décrites dans le Tableau II.

Tableau II. Différentes techniques pour la détection de *Cryptosporidium*

Techniques		Exemples	Description	Avantages	Désavantages
Microscopie	Colorations	Ziehl–Neelsen,	Oocystes apparaissent en rouge sur fond vert ou bleu et mesurant entre 5 et 6 µm de diamètre Oocystes peuvant présenter une vacuole apparaissant comme une zoneplus claire	• Largement disponible • Rapide • Spécifique • Faible coût des réactifs	• Faible sensibilité • Nécessité d'effectuer la lecture au moins à l'objectif 40 • Nécessite des techniciens qualifiés pour l'observation
		Auramine-phenol	Les parasites apparaissent jaune verdâtre brillant sur fond foncé	• Rapide • Spécifique • Sensible • Permet la différenciation entres les oocystes pleins et vides	• Coûteux • Nécessite de la microscopie à fluorescence • Requiert l'utilisation de produits toxiques

		Heine	Les oocystes apparaissent très réfringents, non colorés avec un point sombre au centre, sur un fond rouge	• Sensible	• Nécessite de la microscopie à contraste de phase • La réfringence ne dure qu'une quinzaine de minutes.
Immunologie	Identification du parasite	Anticorps immuno- fluorescent (IFA)	Permet l'identification des oocystes par un anticorps monoclonal et une révélation par fluorescence. Oocystes marqués avec une fluorescence verte périphérique (paroi de l'oocyste)	• Bonne sensibilité • Bonne spécificité • Balayage rapide des oocystes sous faible grossissement • Intéressant pour le diagnostic des pauci-infestations • Intéressant pour les études épidémiologiques	• Coûteux • Nécessite de la microscopie à fluorescence • Relativement long
	Détection de coproantigènes dans les selles	Enzyme linked immunosorbent assay (ELISA)		• Technique automatisée Utilisée pour les enquêtes de masse • Rapide • Bonne sensibilité • Ne nécessite pas de la concentration de matières fécales avant le traitement des échantillons • • Bonne spécificité	• Coûteux • Équipement indispensable • Sensibilité et spécificité dépendants de la quantité d'oocystes

				• Simple • Automatisable	
Moléculaire		PCR nichée	Utilise deux paires d'amorces pour amplifier une partie d'un gène	• Excellente sensibilité • Excellente sensibilité • Technique automatisée et reproductible • Permet la détermination de l'espèce et du subtype après séquençage des produits de PCR • Permet la quantification • Possibilité de combiner en multiplex pour des entéropathogènes supplémentaires	• Instrumentation coûteuse • Techniquement exigeant • Besoin de techniciens qualifiés • Technique ne fournissant pas de données sur la viabilité et l'infectiosité du parasite

Ainsi, l'examen microscopique est utile pour rechercher la présence d'oocystes dans les différents types d'échantillons. Cependant, les oocystes ne sont pas facilement identifiables lors d'un examen parasitologique classique et une recherche en utilisant des techniques de coloration spécifiques est conseillée pour l'observation microscopique. Par conséquent, le clinicien doit faire une ordonnance spécifique (56).

Différentes techniques de coloration permettent de détecter *Cryptosporidium* spp. à partir des différents prélèvements (selles, liquide d'aspiration duodénales, biopsies, liquide de lavage bronchoalveolaire, etc.). La technique de référence est la coloration de Zielh-Neelsen modifiée sur frottis obtenus directement ou après techniques de concentration. Cette technique est basée sur le fait que les oocystes peuvent être colorés en rouge avec de la carbo-fuchsine alors que les selles par contre-coloration au vert malachite apparaîtront vert ou bleu (Figure 2) (56). Cependant, d'autres techniques de coloration (fluorescentes ou non) sont disponibles ; parmi elles, la technique de Heine basée sur les propriétés réfringentes des oocystes apparaît bien plus rapide et sensible que la coloration de Ziehl-Neelsen laissant suggérer une évolution probable de la technique de référence microscopique (Figure 2). De plus, l'hématoxyline et l'éosine peuvent être utilisés pour la confirmation histologique du diagnostic lorsque des biopsies sont disponibles.

Des techniques immunologiques ont aussi été mises en place pour le diagnostic de *Cryptosporidium*. Une capture de copro-antigènes est possible par la technique ELISA ou par un marquage des cryptosporidies par un anticorps spécifique couplé à un fluorochrome (Figure 2) (IFA). Toutefois, l'avènement des techniques moléculaires a permis d'obtenir une meilleure sensibilité. Ces techniques (telles que la PCR nichée ou la qPCR, la PCR-RFLP, l'analyze microsatellite, etc.) permettent aussi d'identifier les différentes espèces et sous-types de *Cryptosporidium* (57).

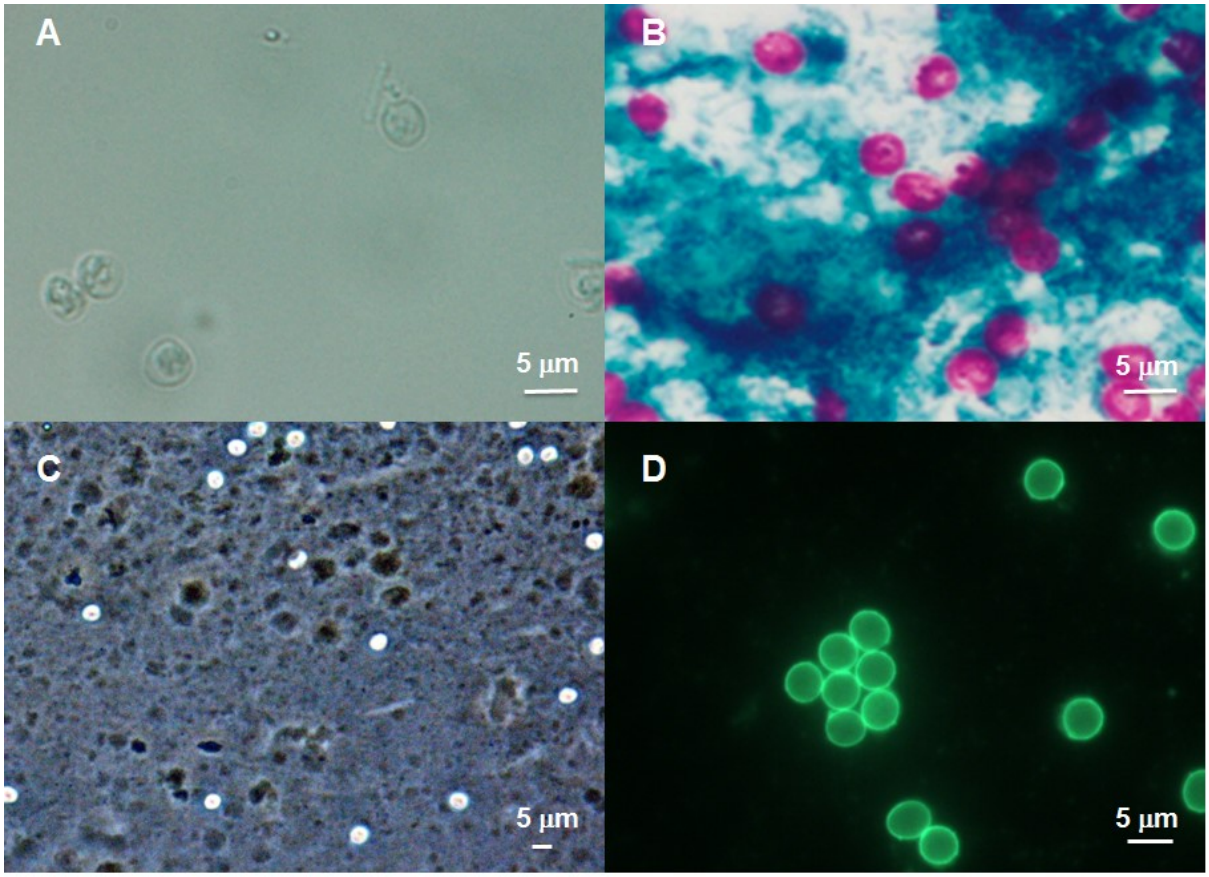


Figure. 2. Oocystes de Cryptosporidium observés en microscopie. (A) Oocystes observés en microscopie à contraste interférentiel après séparation immunomagnétique. (B) Coloration de Ziehl-Neelsen modifiée. (C) Coloration de Heine. (D) Oocystes marqués par un anticorps spécifique couplé à un fluorochrome.

La quantité et la qualité de l'échantillon ainsi que la quantité d'oocystes ou d'antigènes sont naturellement des facteurs essentiels qui détermineront le succès de la méthode de détection. Pour un diagnostic précis, plusieurs examens d'échantillons sont nécessaires, en particulier dans le cas d'infections subcliniques. Les oocystes étant excrétés par intermittence, l'idéal est d'examiner si possible trois échantillons prélevés un jour sur deux.

Traitement et nécessité de développement de molécules efficaces

Les médicaments utilisés contre les protozoaires apicomplexes se sont révélés inefficaces contre *Cryptosporidium* sp. ce qui peut être dû à sa localisation particulière dans la vacuole parasitophore. Les voies d'absorption des nutriments et des médicaments par *Cryptosporidium* sp. sont encore mal connues mais l'activité des médicaments anti-cryptosporidiens peut dépendre de leur transport soit directement à travers la membrane de la cellule hôte à partir de

la lumière intestinale soit indirectement via le cytoplasme de cette cellule (58). Chez les patients atteints de SIDA pouvant bénéficier d'un traitement antirétroviral, le risque de cryptosporidiose a été fortement diminué de près de 96% par l'utilisation d'inhibiteurs de protéase du VIH. L'activité anticryptosporidienne de nombreux inhibiteurs de protéase du VIH (indinavir, saquinavir et ritonavir) suggère un bénéfice thérapeutique direct (59).

Le médicament anticryptosporidien idéal doit être peu coûteux, sûr, avec un schéma posologique court (< 7 jours), disponible par voie orale avec une formulation spécifique pour les enfants en bas âge (0 à 24 mois) (60). Il doit en outre être efficace à la fois contre *C. parvum* et *C. hominis* chez le patient immunodéprimé et l'enfant malnutri. Bien que son administration par voie orale soit souhaitable pour traiter une cryptosporidiose intestinale, il n'est pas clairement établi qu'une bonne biodisponibilité intestinale du médicament doive être privilégiée par rapport à une bonne biodisponibilité systémique.

Médicaments actuellement disponibles contre la cryptosporidiose

Actuellement, un seul médicament, le nitazoxanide (NTZ), chef de file de la famille des thiazolides, a été validé par la FDA (Food and Drug Administration) aux Etats-Unis avec comme indication, le traitement de la cryptosporidiose de l'immunocompétent et n'est pas loin d'être l'anticryptosporidien idéal. Des essais cliniques randomisés ont montré des taux de guérison clinique et parasitologique (jusqu'à 93%) significativement plus élevés par rapport au placebo (30-40%) chez les adultes immunocompétents et les enfants âgés de plus d'un an traités par 3 jours de NTZ (61). Au cours des 20 dernières années, ont été accumulées des données sur la sécurité et l'efficacité du NTZ chez des sujets immunocompétents infectés. Plus de 70 millions de personnes ont été traitées en Amérique du Nord et du Sud sans aucun événement indésirable significatif signalé. Le NTZ n'inhibe pas de façon significative les enzymes du cytochrome P450, ne provoquant aucune interaction médicamenteuse ni aucune interférence avec la warfarine. Aucune cardiotoxicité n'a été observée chez des volontaires sains et le NTZ n'est ni embryotoxique ni tératogène chez le rat et le lapin. En revanche, le NTZ ne s'est pas avéré plus efficace que le placebo chez les patients atteints de SIDA et, chez des enfants malnutris, malgré une diminution de la mortalité liée à la parasitose (18% du groupe placebo sont décédés au jour 8 vs 0% du groupe traité), le NTZ n'a guéri que 56% des enfants malnutris (62). D'autres médicaments disponibles ont été utilisés mais n'ont pas été validés pour le traitement de la cryptosporidiose. La paromomycine est un aminoglycoside oral, non résorbé par la muqueuse qui reste concentré dans la lumière intestinale. L'utilisation de la

paromomycine dans le traitement de la cryptosporidiose chez des patients infectés par le VIH n'a pas montré de différence significative avec le placebo (63). L'azithromycine est un antibiotique macrolide dont l'association avec le NTZ a permis l'amélioration des symptômes diarrhéiques chez les porcelets gnotobiotiques infectés par *C. hominis* sans toutefois inhiber complètement l'excrétion des oocystes (64). Cette association est assez couramment utilisée en cas de cryptosporidiose chez les patients immunodéprimés sans qu'un réel avantage ait pu être clairement démontré (65).

Nouvelle indication pour des médicaments existants

Chez *Cryptosporidium* spp., il n'existe pas de synthèse endogène d'isopentényl-pyrophosphate (IPP), précurseur des isoprénoïdes, impliqués dans divers processus cellulaires essentiels pour le parasite qui ne peut qu'utiliser celui de l'hôte. Les statines, notamment l'itavastatine et l'atorvastatine, inhibiteurs de la 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) réductase humaine et donc de la synthèse des isoprénoïdes, bloquent la croissance de *C. parvum*. Les statines sont largement utilisées en médecine humaine pour réduire la cholestérolémie et peuvent donc interférer avec la production de lipides essentiels pour *Cryptosporidium* (66). Il a de plus été démontré que l'atorvastatine avait une activité synergique avec le NTZ tant au niveau de l'excrétion des oocystes qu'au niveau de l'amélioration des altérations histologiques chez des souris immunodéprimées par la dexamethasone infectées par *Cryptosporidium* (67). La clofazimine, un médicament essentiel de l'OMS pour le traitement de la lèpre a été identifié après criblage systématique de nombreuses molécules, comme étant très actif contre *Cryptosporidium in vitro* et *in vivo* dans un modèle de souris IFN-□KO (68). Un essai contrôlé randomisé (ClinicalTrials.gov NCT03341767) chez les patients infectés par le VIH et co-infectés par *Cryptosporidium* est en cours. Une étude a révélé que la vectorisation de la clofazimine, faiblement soluble, avec des nanoparticules améliorait son efficacité (68).

Nouvelles cibles et médicaments en développement

Le criblage de composés issues de la « Malaria Box » a révélé qu'un composé à base de piperazine, le MMV665917, était très actif contre *C. hominis* et *C. parvum*. MMV665917, dont la cible parasitaire est inconnue, s'est avérée très efficace dans un modèle d'infection aiguë chez la souris IFN-□KO et d'infection chronique chez des souris NOD SCID Gamma (NSG). Une action parasiticide de cette molécule, contrairement à la paromomycine, au NTZ et à la clofazimine, est suggérée par les données *in vitro* (69). En l'absence d'un système immunitaire compétent, l'efficacité d'une molécule anti-cryptosporidienne pourrait dépendre de cette

activité parasiticide. L'activité parasitostatique du NTZ pourrait en partie expliquer son inefficacité en cas d'immunodépression profonde. Les protéines kinases dépendantes du calcium sont essentielles au parasite au cours des processus d'invasion de la cellule hôte. Des inhibiteurs de protéine kinases (BKI pour « bumped kinase inhibitors ») ont été développés et certains d'entre eux ont permis d'améliorer la diarrhée chez les veaux infectés et ont réduit la charge parasitaire et la sévérité de la maladie dans le modèle de porcelet gnotobiotique infecté par *C. hominis*. L'efficacité *in vivo* de ces inhibiteurs est corrélée à leurs concentrations obtenues dans les intestins (70). Le criblage de pyrazolopyridines, connues pour inhiber PI(4)K8, une lipide kinase de *Plasmodium*, a conduit à l'identification de KDU731, inhibiteur de l'activité enzymatique de PI4K de *Cryptosporidium* sp. Le KDU731 dont l'évaluation préclinique est en cours a considérablement réduit l'excrétion des oocystes chez les souris (IFN)-γ KO infectées avec une disparition complète des parasites dans les intestins des animaux traités. Enfin, le traitement par KDU731 chez les veaux a considérablement réduit l'excrétion des oocystes et la diarrhée dans les 3 jours suivant le traitement (71). Une autre voie essentielle pour *Cryptosporidium* sp. est la synthèse des purines et 8 inhibiteurs de l'inosine monophosphate déshydrogenase de *C. parvum*, nécessaire pour la conversion de l'adénosine en guanine se sont avérés efficaces dans un modèle de souris IL-12 KO. Parmi ceux-ci, le P131 administré quotidiennement pendant 3 jours a donné de meilleurs résultats que la paromomycine (72).

Cryptosporidiose extra-intestinale

Dans le contexte de la cryptosporidiose extra-intestinale, des médicaments agissant sur tous les sites d'infection et ayant une biodisponibilité systémique seraient nécessaires. L'association d'atorvastatine et de NTZ a montré une amélioration notable des hémorragies et de l'inflammation interstitielle pulmonaire par rapport à une amélioration nulle à partielle avec chacune d'entre elles (67). La sévérité des signes cliniques pourraient justifier une thérapie spécifique par voie parentérale et l'aminoxanide (RM-5061), un nouveau dérivé d'amino-ester thiazolide et première forme soluble de NTZ, va être évaluée dans une étude clinique de phase I et pourrait être candidat pour le premier traitement injectable de la cryptosporidiose (73). Finalement, pour tous ces composés qui ont déjà prouvé leur efficacité dans des modèles animaux, le défi consiste maintenant à sélectionner les meilleurs candidats pour des études cliniques.

Introduction

Prévention de la cryptosporidiose

Les options thérapeutiques pour prévenir et traiter la cryptosporidiose étant limitées, les moyens pour combattre la maladie reposent essentiellement sur la prévention. Les mesures de lutte contre le péril fécal s'appliquent pour éviter l'infection par *Cryptosporidium* sp. et limiter sa propagation. L'éducation sanitaire est donc la principale mesure préventive.

Sur le plan individuel (Tableau III), la prévention consiste à réduire l'exposition au parasite en respectant des règles d'hygiène et en évitant l'ingestion d'eau ou d'aliments pouvant être souillés par des matières fécales. Il est fondamental de se laver les mains avec de l'eau et du savon aussi souvent que nécessaire, après passage aux toilettes, après avoir changé une couche, après un contact avec des animaux, après avoir jardiné ou avant de manipuler et de consommer des aliments. Il faut éviter le contact avec des individus malades ainsi que les jeunes animaux (veaux notamment), *a fortiori* s'ils sont diarrhéiques. Il est nécessaire avant leur consommation de laver à l'eau potable tout fruit et légume pouvant être souillé par de la terre ou des selles ou encore par de l'eau d'arrosage ou d'irrigation provenant d'une ressource hydrique contaminée. Il est aussi préconisé d'éviter la consommation de coquillages crus si ceux-ci sont en provenance d'un lieu non identifié ou non autorisé (pêche à pied familiale par exemple). Il est recommandé de boire uniquement de l'eau connue pour ne pas être contaminée et d'éviter la consommation d'eau de surface susceptible d'être souillée par des fèces d'animaux (pour les randonneurs et campeurs). En cas d'incertitude, il faut faire bouillir l'eau, y compris celle qui est utilisée pour se rincer les dents ou pour laver les aliments. De façon générale, il est recommandé d'éviter les bains en *Plasmodium falciparum* dehors des endroits prévus à cet effet et qui font l'objet de contrôles microbiologiques. Il faut éviter d'avaler de l'eau lors des baignades en rivière, dans un lac ou même en piscine. Il faut ici rappeler qu'en raison de leur grande résistance au chlore, les oocystes de *Cryptosporidium* peuvent représenter un danger sanitaire lié aux piscines. Il est donc préconisé aux personnes ayant contractées une cryptosporidiose dans les 15 jours précédents de ne pas se baigner dans des eaux de loisirs.

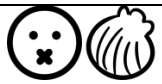
Cryptosporidium sp. peut infecter n'importe quel individu mais certaines personnes sont plus vulnérables ou plus exposées au risque. Chez les sujets fortement immunodéprimés, la prévention conduit à recommander exclusivement la consommation d'eau embouteillée. De même, la consommation de glaçons préparés avec de l'eau du robinet n'est pas recommandée. Il leur est préconisé de cuire ou d'éplucher les fruits et légumes et de ne pas consommer de lait et de jus de fruits non pasteurisés. Certains individus, de par leur profession, sont soumis à un risque de forte exposition comme les personnels travaillant dans les centres de soins, les crèches, les élevages, les abattoirs ou dans l'assainissement des eaux usées. La formation et

l'information du personnel doit faire partie d'un plan de gestion du risque infectieux qui doit prévoir le port de protections individuelles (gants, blouses) et des procédures spécifiques (nettoyage, désinfection, gestion des déchets…). Dans les centres de soins et les élevages, il parait préférable d'isoler les individus ou animaux malades afin d'enrayer la dynamique de transmission et ainsi éviter l'infection d'autres sujets potentiellement réceptifs.

La prévention collective consiste à contrôler et à protéger les ressources d'eau destinée à la consommation humaine de toute contamination fécale et à s'assurer de la protection et de l'intégrité des réseaux de distribution ainsi que de la qualité de l'eau distribuée. La prévention de la contamination environnementale par des excrétas d'origine humaine repose sur la maîtrise des rejets d'eaux en sortie de stations d'épuration ainsi que sur celle de l'épandage des boues qui y sont produites. Il en va de même pour le stockage et le traitement des effluents d'élevage qui doivent se faire en respect de la réglementation en vigueur. La prévention collective du risque lié à la contamination des aliments repose avant tout sur l'application des réglementations existantes sur la protection des zones de production.

Tableau III. Mesures d'hygiène individuelles pour éviter la cryptosporidiose

Source de pictogrammes : https://fr.freepik.com/photos-vecteurs-libre/banner">Banner vecteur créé par pch.vector - fr.freepik.com

Mesures individuelles	
	Se laver les mains
	Eviter le contact avec des individus ou des animaux malades
	Éviter le contact avec des selles humaines (couches), des objets et des linges souillés par des selles
	Laver à l'eau potable tout fruit et légume avant sa consommation
	Eviter la consommation de coquillages crus
	Boire de l'eau connue pour ne pas être contaminée

	Eviter les bains en dehors des endroits prévus à cet effet
	Eviter d'avaler de l'eau lors des baignades en rivière, dans un lac ou même en piscine
Mesures complémentaires pour les patients immunodéprimés	
	Port de protections individuelles (gants, blouses) et procédures spécifiques si risque de forte exposition
	Consommation d'eau embouteillée
	Cuire ou éplucher les fruits et légumes
	Eviter la consommation de glaçons préparés avec de l'eau du robinet
	Eviter la consommation de lait et de jus de fruits non pasteurisés

Conclusions

La cryptosporidiose est considérée comme l'une des principales causes de diarrhées parasitaires chez l'Homme. Cette infection touche autant les personnes immunocompétentes que les immunodéprimées mais l'infection s'avère en général plus sévère chez ces derniers ainsi que chez les enfants de moins de 5 ans.

Les oocystes peuvent survivre très longtemps dans l'environnement et résistent à la plupart des désinfectants, ce qui rend difficile leur élimination. Les données de prévalence sont encore sous-estimées en raison de l'absence de symptômes pathognomoniques et d'un manque d'outils de laboratoire dans les panels de diagnostic en routine.

Des différences dans les manifestations cliniques dues à la cryptosporidiose ont été observées entre différentes espèces, génotypes et sous-types de *Cryptosporidium* sp.. Les signes et symptômes dépendent également de l'état nutritionnel et immunitaire de l'hôte et des facteurs de virulence du parasite. De nouvelles études utilisant des méthodes moléculaires sont nécessaires pour mieux caractériser la pathogenèse de l'infection.

L'ensemble des données expérimentales chez l'animal et clinico-épidémiologiques chez l'Homme suggère fortement que le spectre de pathogénicité de *Cryptosporidium* sp. comporte

un pouvoir carcinogène qui s'exprime dans le tractus gastro-intestinal de mammifères immunodéprimés et probablement de l'Homme. Malgré l'importance que semble avoir cet agent infectieux en santé humaine et animale et le nombre croissant de preuves de son lien avec la pathologie cancéreuse, l'intérêt de la communauté scientifique pour son rôle et pour l'étude des mécanismes par lesquels il induirait le développement de cancer reste insuffisant. D'autant que nous savons aujourd'hui que nous pourrions remédier à un bon nombre de cancers simplement en prévenant les infections.

Ils existent plusieurs méthodes de détection du parasite même si l'infection reste largement sous-diagnostiquée. L'examen microscopique des selles peut être considéré comme la technique la plus simple à réaliser, peu onéreuse et ayant de bonnes performances. Dans les milieux à faibles ressources où les taux d'infections mixtes sont élevés, la mise en place de méthodes de diagnostic quantitatifs serait prioritaire.

Les options de traitement de la cryptosporidiose sont limitées. De nouvelles alternatives sont donc nécessaires et les efforts visant à trouver de nouveaux traitements doivent répondre aux besoins cliniques, principalement ceux des patients immunodéprimés et des enfants malnutris pour lesquels l'efficacité du NTZ reste limitée. La prévention pour réduire les facteurs de risque serait l'intervention la plus importante.

A l'heure actuelle des outils nécessaires pour poursuivre des études génomiques et post-génomiques sont disponibles. Celles-ci permettront, à n'en pas douter, non seulement d'apporter des connaissances supplémentaires sur la biologie de *Cryptosporidium* sp. mais également d'identifier de nouvelles cibles thérapeutiques pour lutter contre ce parasite.

La disponibilité et l'analyze des différents génomes de *Cryptosporidium* sp. offrent des perspectives d'études sans précédent sur la biologie de ces parasites, leur spécificité d'hôte, leur pathogénicité, leur évolution et leur virulence. Pourtant, les études de génomique comparative de *Cryptosporidium* sp., dont il n'existe toujours pas de système de culture continue, restent à ce jour assez limitées. Très certainement, la démocratisation du séquençage à haut-débit et les nouveaux outils ciblés d'édition du génome comme CRISPR/Cas9, récemment décrits (35,36), permettront de stimuler les recherches sur ce parasite, de confirmer les résultats de ces analyzes génomiques et d'aborder l'analyze fonctionnelle des gènes d'intérêt du parasite ainsi découverts.

Références bibliographiques

(1) Tzipori S, Widmer G. A hundred-year retrospective on cryptosporidiosis. *Trends Parasitol* 2008 ; **24** 184–9.

(2) Baldursson S, Karanis P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks–an update 2004–2010. *Water Res* 2011 ; **45** : 6603–14.

(3) Ramirez NE, Ward LA, Sreevatsan S. A review of the biology and epidemiology of cryptosporidiosis in humans and animals. *Microbes Infect* 2004 ; **6** : 773–85.

(4) Mac Kenzie WR, Hoxie NJ, Proctor ME, Gradus MS, Blair KA, Peterson DE *et al.* A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply. *N Engl J Med* 1994 ; **331** : 161–7.

(5) Smith A, Reacher M, Smerdon W, Adak GK, Nichols G, Chalmers RM. Outbreaks of waterborne infectious intestinal disease in England and Wales, 1992-2003. *Epidemiol Infect* 2006 ; **134** : 1141–9.

(6) Beaudeau P, De Valk H, Vaillant V, Mannschott C, Tillier C, Mouly D *et al.* Lessons learned from ten investigations of waterborne gastroenteritis outbreaks, France, 1998-2006. *J Water Health* 2008 ; **6** : 491–5.

(7) Bouwknegt M, Devleesschauwer B, Graham H, Robertson LJ, van der Giessen JWB, Akkari H *et al.* Prioritisation of food-borne parasites in Europe, 2016. *Eurosurveillance* 2018 ; **23** . doi:10.2807/1560-7917.ES.2018.23.9.17-00161.

(8) Savioli L, Smith H, Thompson A. *Giardia* and *Cryptosporidium* join the 'Neglected Diseases Initiative'. *Trends Parasitol* 2006 ; **22** : 203–8.

(9) Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S *et al.* Burden and etiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* 2013 ; **382** : 209–22.

(10) Delahoy MJ, Omore R, Ayers TL, Schilling KA, Blackstock AJ, Ochieng JB *et al.* Clinical, environmental, and behavioral characteristics associated with *Cryptosporidium* infection among children with moderate-to-severe diarrhea in rural western Kenya, 2008–2012: The Global Enteric Multicenter Study (GEMS). *PLoS Negl Trop Dis* 2018 . doi:10.1371/journal.pntd.0006640.

(11) Lendner M, Daugschies A. *Cryptosporidium* infections: Molecular advances. *Parasitology* 2014 ; **141** : 1511–32.

(12) O'Hara SP, Chen XM. The cell biology of *Cryptosporidium* infection. *Microbes Infect* 2011 ; **13** : 721–30.

(13) Barta JR, Thompson ARC. What is *Cryptosporidium*? Reappraising its biology and phylogenetic affinities. *Trends Parasitol* 2006 ; **22** : 463–8.

(14) Carreno RA, Matrin DS, Barta JR. *Cryptosporidium* is more closely related to the gregarines than to coccidia as shown by phylogenetic analysis of apicomplexan parasites inferred using small-subunit ribosomal RNA gene sequences. *Parasitol Res* 2002 ; **85** : 899–904.

(15) Aldeyarbi HM, Karanis P. The fine structure of sexual stage development and sporogony of *Cryptosporidium parvum* in cell-free culture. *Parasitology* 2016 ; **143** : 749–61.

(16) Ryan U, Paparini A, Monis P, Hijjawi N. It's official – *Cryptosporidium* is a gregarine: What are the implications for the water industry? *Water Res* 2016 ; **15** : 305–13.

(17) Clode PL, Koh WH, Thompson RCA. Life without a Host Cell: What is *Cryptosporidium*? *Trends Parasitol* 2015 ; **31** : 614–24.

(18) Koh W, Thompson A, Edwards H, Monis P, Clode PL. Extracellular excystation and development of *Cryptosporidium*: Tracing the fate of oocysts within Pseudomonas aquatic biofilm systems. *BMC Microbiol* 2014 ; **14:281** . doi:10.1186/s12866-014-0281-8.

(19) Borowski H, Thompson RCA, Armstrong T, Clode PL. Morphological characterization of *Cryptosporidium parvum* life-cycle stages in an in vitro model system. *Parasitology* 2010 ; **137** : 13–26.

(20) Cavalier-Smith T. Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of Sporozoa. *Eur J Protistol* 2014 ; **50** : 472–95.

(21) Squire SA, Ryan U. *Cryptosporidium* and *Giardia* in Africa: current and future challenges. Parasites and Vectors. 2017 ; **10** . doi:10.1186/s13071-017-2111-y.

(22) Slapeta J. Cryptosporidiosis and *Cryptosporidium* species in animals and humans: A thirty colour rainbow? *Int J Parasitol* 2013 ; **43** : 957–70.

(23) Zahedi A, Monis P, Aucote S, King B, Paparini A, Jian F *et al.* Zoonotic *Cryptosporidium* species in animals inhabiting sydney water catchments. *PLoS One* 2016 ; **11** : e0168169.

(24) Ryan U, Fayer R, Xiao L. *Cryptosporidium* species in humans and animals: current understanding and research needs. *Parasitology* 2014 ; **141** : 1667–85.

(25) Nader JL, Mathers TC, Ward BJ, Pachebat JA, Swain MT, Robinson G *et al.* Evolutionary genomics of anthroponosis in *Cryptosporidium*. *Nat Microbiol* 2019 ; **4** : 826–36.

(26) Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA *et al.* Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*. *Science* 2004 ; **304** : 441–5.

(27) Lippuner C, Ramakrishnan C, Basso WU, Schmid MW, Okoniewski M, Smith NC *et al.* RNA-Seq analysis during the life cycle of *Cryptosporidium parvum* reveals significant differential gene expression between proliferating stages in the intestine and infectious sporozoites. *Int J Parasitol* 2018 ; **48** : 413–22.

(28) Matos LVS, McEvoy J, Tzipori S, Bresciani KDS, Widmer G. The transcriptome of *Cryptosporidium* oocysts and intracellular stages. *Sci Rep* 2019 ; **9** : 7856.

(29) Mele R, Gomez Morales MA, Tosini F, Pozio E. *Cryptosporidium parvum* at different developmental stages modulates host cell apoptosis in vitro. *Infect Immun* 2004 ; **72** : 6061–7.

(30) Ming Z, Gong AY, Wang Y, Zhang XT, Li M, Dolata CE *et al.* Trans-suppression of defense DEFB1 gene in intestinal epithelial cells following *Cryptosporidium parvum* infection is associated with host delivery of parasite Cdg7_FLc_1000 RNA. *Parasitol Res* 2018 ; **117** : 831–40.

(31) Okhuysen PC, Chappell CL, Crabb JH, Sterling CR, DuPont HL. Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. *J Infect Dis* 2002 ; **180** : 1275–81.

(32) Chappell CL, Okhuysen PC, Langer-Curry R, Widmer G, Akiyoshi DE, Tanriverdi S *et al. Cryptosporidium hominis*: Experimental challenge of healthy adults. *Am J Trop Med Hyg* 2006 ; **75** : 851–7.

(33) Certad G, Viscogliosi E, Chabé M, Cacciò SM. Pathogenic mechanisms of *Cryptosporidium* and *Giardia*. *Trends Parasitol* 2017 ; **33** : 561–76.

(34) Di Genova BM, Tonelli RR. Infection strategies of intestinal parasite pathogens and host cell responses. *Front Microbiol* 2016 ; **7** : doi: 10.3389.

(35) Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y *et al.*

Genetic modification of the diarrheal pathogen *Cryptosporidium parvum*. *Nature* 2015 ; **523** : 477–80.

(36) Sateriale A, Slapeta J, Baptista R, Engiles JB, Gullicksrud JA, Herbert GT *et al.* Protective immunity in a genetically tractable natural mouse model of cryptosporidiosis. *Cell Host Microbe* 2019 ; **26** : 135–46.

(37) Bouzid M, Hunter PR, Chalmers RM, Tyler KM. *Cryptosporidium* pathogenicity and virulence. *Clin Microbiol Rev* 2013 ; **26** : 115–34.

(38) King P, Tyler KM, Hunter PR. Anthroponotic transmission of *Cryptosporidium parvum* predominates in countries with poorer sanitation: a systematic review and meta-analysis. *Parasit Vectors* 2019 ; **12** . doi:10.1186/s13071-018-3263-0.

(39) Cacciò SM, Chalmers RM. Human cryptosporidiosis in Europe. Clin. Microbiol. Infect. 2016 ; **22** : 471–80.

(40) Costa D, Razakandrainibe R, Sautour M, Valot S, Basmaciyan L, Gargala G *et al.* Human cryptosporidiosis in immunodeficient patients in France (2015–2017). *Exp Parasitol* 2018 ; **192** : 108–12.

(41) Ripert C, Guyot K. Cryptosporidiose. In: *Epidémiologie des maladies parasitaires*. Edition Médicales Internationales: Cachan, 2003, pp 269–97.

(42) Marcos LA, Gotuzzo E. Intestinal protozoan infections in the immunocompromised host. *Curr Opin Infect Dis* 2013 ; **26** : 295–301.

(43) Hunter PR, Nichols G. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. *Clin Microbiol Rev* 2002 ; **15** : 145–54.

(44) Lilja M, Widerström M, Lindh J. Persisting post-infection symptoms 2 years after a large waterborne outbreak of *Cryptosporidium hominis* in northern Sweden. *BMC Res Notes* 2018 ; **11** : 10.1186/s13104-018-3721-y.

(45) Cama VA, Ross JM, Crawford S, Kawai V, Chavez-Valdez R, Vargas D *et al.* Differences in clinical manifestations among *Cryptosporidium* species and subtypes in HIV-infected persons. *J Infect Dis* 2007 ; **196** : 684–91.

(46) Jacqueline C, Tasiemski A, Sorci G, Ujvari B, Maachi F, Missé D *et al.* Infections and cancer: The ‘fifty shades of immunity’ hypothesis. *BMC Cancer* 2017 ; **17** : doi: 10.1186/s12885-017-3234-4.

(47) Shield KD, Marant Micallef C, de Martel C, Heard I, Megraud F, Plummer M *et al.* New cancer cases in France in 2015 attributable to infectious agents: a systematic

review and meta-analysis. *Eur J Epidemiol* 2018 ; **33** : 263–74.

(48) Hassanein SMA, Abd-El-Latif MMS, Hassanin OM, Abd-El-Latif LMS, Ramadan NI. *Cryptosporidium* gastroenteritis in Egyptian children with acute lymphoblastic leukemia: Magnitude of the problem. *Infection* 2012 ; **40** : 279–84.

(49) Benamrouz S, Conseil V, Creusy C, Calderon E, Dei-Cas E, Certad G. Parasites and malignancies, a review, with emphasis on digestive cancer induced by *Cryptosporidium parvum*(Alveolata: Apicomplexa). *Parasite* 2012 ; **19** : 101–15.

(50) Sulżyc-Bielicka V, Kołodziejczyk L, Jaczewska S, Bielicki D, Safranow K, Bielicki P *et al.* Colorectal cancer and *Cryptosporidium* spp. infection. *PLoS One* 2018 ; **13** : e0195834.

(51) Osman M, Benamrouz S, Guyot K, Baydoun M, Frealle E, Chabe M *et al.* High association of *Cryptosporidium* spp. infection with colon adenocarcinoma in Lebanese patients. *PLoS One* 2017 ; **12** : e0189422.

(52) Essid R, Menotti J, Hanen C, Aoun K, Bouratbine A. Genetic diversity of *Cryptosporidium* isolates from human populations in an urban area of Northern Tunisia. *Infect Genet Evol* 2018 ; **58** : 237–42.

(53) Kopacz Ż, Kváč M, Karpiński P, Hendrich AB, Sąsiadek MM, Leszczyński P *et al.* The First evidence of *Cryptosporidium meleagridis* infection in a colon adenocarcinoma from an immunocompetent patient. *Front Cell Infect Microbiol* 2019 ; **9** : doi: 10.3389/fcimb.2019.00035.

(54) Baydoun M, Vanneste SB, Creusy C, Guyot K, Gantois N, Chabe M *et al.* Three-dimensional (3D) culture of adult murine colon as an in vitro model of cryptosporidiosis: Proof of concept. *Sci Rep* 2017 ; **7** : 17288.

(55) Benamrouz S, Conseil V, Chabe M, Praet M, Audebert C, Blervaque R *et al.* *Cryptosporidium parvum*-induced ileo-caecal adenocarcinoma and Wnt signaling in a mouse model. *Dis Model Mech* 2014 ; **7** : 693–700.

(56) Guyot K, Sarfati C, Derouin F. Actualités sur l'épidémiologie et le diagnostic de la cryptosporidiose. *Feuill Biol* 2012 ; **LIII** : 21–9.

(57) Checkley W, White AC, Jaganath D, Arrowood MJ, Chalmers RM, Chen X-M *et al.* A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *Lancet Infect Dis* 2015; **15** : 85–94.

(58) Ehrenman K, Wanyiri JW, Bhat N, Ward HD, Coppens I. *Cryptosporidium parvum*

scavenges LDL-derived cholesterol and micellar cholesterol internalized into enterocytes. *Cell Microbiol* 2013 ; **15** : 1182–97.

(59) Mele R, Gomez Morales MA, Tosini F, Pozio E. Indinavir reduces *Cryptosporidium parvum* infection in both *in vitro* and *in vivo* models. *Int J Parasitol* 2003 ; **33** : 757–64.

(60) Huston CD, Spangenberg T, Burrows J, Willis P, Wells TNC, van Voorhis W. A proposed target product profile and developmental cascade for new cryptosporidiosis treatments. *PLoS Negl Trop Dis* 2015 ; **9** : e0003987.

(61) Rossignol JF, Kabil SM, El-gohary Y, Younis AM. Effect of nitazoxanide in diarrhea and enteritis caused by *Cryptosporidium* species. *Clin Gastroenterol Hepatol* 2006 ; **4** : 320–4.

(62) Amadi B, Mwiya M, Musuku J, Watuka A, Sianongo S, Ayoub A *et al.* Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: A randomised controlled trial. *Lancet* 2002 ; **360** : 1375–80.

(63) Hewitt RG, Yiannoutsos CT, Higgs ES, Carey JT, Geiseler PJ, Soave R *et al.* Paromomycin: No more effective than placebo for treatment of cryptosporidiosis in patients with advanced Human Immunodeficiency Virus infection. AIDS Clinical Trial Group. *Clin Infect Dis* 2002 ; **31** : 1084–92.

(64) Lee S, Harwood M, Girouard D, Meyers MJ, Campbell MA, Beamer G *et al.* The therapeutic efficacy of azithromycin and nitazoxanide in the acute pig model of *Cryptosporidium hominis*. *PLoS One* 2017 ; **12** : e0185906.

(65) Lanternier F, Amazzough K, Favennec L, Mamzer-Bruneel MF, Abdoul H, Tourret J *et al. Cryptosporidium* spp. infection in solid organ transplantation: the nationwide 'TRANSCRYPTO' study. *Transplantation* 2017 ; **101** : 826–30.

(66) Bessoff K, Sateriale A, Lee KK, Huston CD. Drug repurposing screen reveals FDA-approved inhibitors of human HMG-CoA reductase and isoprenoid synthesis that block *Cryptosporidium parvum* growth. *Antimicrob Agents Chemother* 2013 ; **57** : 1804–14.

(67) Madbouly Taha N, Salah A. Yousof HA, El-Sayed SH, Younis AI, Ismail Negm MS. Atorvastatin repurposing for the treatment of cryptosporidiosis in experimentally immunosuppressed mice. *Exp Parasitol* 2017 ; **181** : 57–69.

(68) Zhang Y, Feng J, McManus SA, Lu HD, Ristroph KD, Cho EJ *et al.* Design and

solidification of fast-releasing clofazimine nanoparticles for treatment of cryptosporidiosis. *Mol Pharm* 2017 ; **14** : 3480–8.

(69) Jumani RS, Bessoff K, Love MS, Miller P, Stebbins EE, Teixeira JE *et al.* A novel piperazine-based drug lead for cryptosporidiosis from the medicines for malaria venture open-access malaria box. *Antimicrob Agents Chemother* 2018 ; **14** : 3480–8.

(70) Arnold SLM, Choi R, Hulverson MA, Schaefer DA, Vinayak S, Vidadala RSR *et al.* Necessity of bumped kinase inhibitor gastrointestinal exposure in treating *Cryptosporidium* infection. *J Infect Dis* 2017 ; **216** : 55–63.

(71) Manjunatha UH, Vinayak S, Zambriski JA, Chao AT, Sy T, Noble CG *et al.* A *Cryptosporidium* PI(4)K inhibitor is a drug candidate for cryptosporidiosis. *Nature* 2017 ; **546** : 376–80.

(72) Gorla SK, McNair NN, Yang G, Gao S, Hu M, Jala VR *et al.* Validation of IMP dehydrogenase inhibitors in a mouse model of cryptosporidiosis. *Antimicrob Agents Chemother* 2014 ; **58** : 1603–14.

(73) Stachulski A V., Swift K, Cooper M, Reynolds S, Norton D, Slonecker SD *et al.* Synthesis and pre-clinical studies of new amino-acid ester thiazolide prodrugs. *Eur J Med Chem* 2017 ; **126** : 154–9.

1.2. Epigenetics in Apicomplexan parasites

1.2.1. Need for epigenetic regulations in Apicomplexa pathogens

Apart from *Cryptosporidium*, the phylum Apicomplexa comprises several well-known genera such as *Eimeria*, *Babesia*, *Theileria*, *Plasmodium,* and *Toxoplasma* which can cause infections with considerable impact on the health of humans as well as animals worldwide (Arisue N and Hashimoto T 2015). Out of these, the most studied genera are *Plasmodium* and *Toxoplasma*. The species *Plasmodium falciparum* is responsible for the severe form of Malaria, a mosquito-borne infectious disease that if not promptly treated may lead to death (Olliaro P 2008). Whereas, *Toxoplasma gondii*, is the only species of the *Toxoplasma* genus responsible for the disease called toxoplasmosis, and is considered to be one of the most common parasitic zoonoses worldwide (Tenter AM *et al,* 2000). *P. falciparum* and *T. gondii* were the first Apicomplexan parasites to be included in the genome projects (Gardner MJ *et al,* 2002) (Kissinger JC *et al,* 2003) followed by *C. parvum* (Abrahamsen MS *et al,* 2004). Whole-genome sequencing of several Apicomplexan including *Toxoplasma*, *Plasmodium, and Cryptosporidium* brought to notice the absence of DNA-binding transcription factors that are well-conserved from yeast through humans. On the contrary, these primitive eukaryotes conserve a large repertoire of chromatin remodeling machinery supporting the idea that epigenetic mechanisms play a key role in their gene regulation events (Templeton TJ *et al,* 2004). With the ease with which *T. gondii* and *P. falciparum* can be genetically manipulated in the laboratory, epigenetic research has made substantial progress in understanding Apicomplexan biology. Even though all the research carried out on these two protozoans cannot apply to other Apicomplexans, they can still be considered as a model to understand the biology of the parasites belonging to the same phylum.

1.2.2. Brief history of epigenetic mechanisms

Epigenetics has been defined by CDC as the study of how one's behavior and environment can cause changes that affect the way genes work. Unlike genetic changes, epigenetic changes are reversible and do not change the DNA sequence, but they can change how one's body reads a DNA sequence. By varying levels of gene expression, the same genome can give rise to a wide variety of phenotypes. Gene expression levels can be altered by DNA methylation, nucleosome remodeling, and covalent modification of histones (https://www.cdc.gov/genomics/disease/epigenetics.html).

Histones have been proposed as a primary constituent of chromatin and an obstacle to gene expression since the 1950s (Stedman E 1950). To counter the repressive nature of the chromatin and allow access to DNA, several post-translational modifications take place on the histone tails, such as phosphorylation, acetylation, methylation, ubiquitination, SUMOylation, and ADP-ribosylation (Hakimi MA and Dietsch KW 2007). The presence of such modifications on one or more histone tails is termed "histone code" which is recognized by other proteins to bring about distinct downstream events such as transcriptional activation (Strahl BD and Allis CD 2000). Thus, the histone code is a result of the biochemical activity of enzymes called "writers" such as kinases, histone lysine-methyltransferases (HKMTs), and histone arginine-methyltransferases (PRMTs) and histone acetyltransferases (HATs). This function is reversed by enzymes called "erasers" such as phosphatases, histone deacetylases (HDACs), and histone lysine demethylases (KDMs) (Hakimi MA and Dietsch KW 2007).

It was not until 1964, that Allfrey *et al* provided evidence showing that acetylation of histones correlated with gene activation (Allfrey VG *et al,* 1964). In 1996, a *Tetrahymena* HAT enzyme was found homologous to a yeast transcriptional adaptor called general control nonderepressible-5 (GCN5), since then the research on this topic has been intensified in other protozoans (Brownell JE *et al,* 1996). Within three years of the discovery of GCN5 as a HAT involved in gene expression, a GCN5 homolog was cloned and characterized in *T. gondii,* (Hettmann C and Soldati D 1999), followed by the discovery of PfGCN5 in *P. falciparum* (Fan Q *et al,* 2004).

1.2.3. Importance of histone lysine methylation

Out of all the histone codes, histone lysine methylation has received considerable attention over the years. Initially, it was regarded as a stable chromatin modification that together with DNA methylation defines epigenetic programs (Bannister AJ *et al,* 2002). But soon after the discovery of KDMs that reversibly remove methyl marks highlighted the dynamic nature of this modification (Trojer P and Reinberg D, 2006). Moreover, deregulation in the activity of the HKMTs, which catalyzes the transfer of methyl group onto the lysine residues of the histone tails, has been correlated with diseases such as cancer (Schneider R *et al,* 2002).

All the known HKMTs, are known to contain a conserved methyltransferase domain termed as SET [**S**u(var)3-9, **E**nhancer-of-zeste, **T**rithorax] domain. This 130 amino acid long domain is evolutionarily conserved from yeast to mammals (Jenuwein T *et al,* 1998). To date, seven main families of HKMTs are known namely the SUV39, SET1, SET2, EZ, RIZ, SMYD, and SUV4-

20. The SET7/9 and SET8 are considered orphan members of the SET-domain proteins superfamily (Dillon SC *et al,* 2005). The SUV39 family was the first HKMT to be characterized. All the homologs belonging to this family specifically methylate lysine 9 of histone 3 (H3 K9) (Rea S *et al,* 2000). The list of substrate specificities of all the characterized HKMTs has been summarized in Table 1.

Table 1 – Substrate specificities of different functionally characterized HKMT families. in Apicomplexa and other eukaryotes

Site specificity	HKMTs families	Apicomplexans	Other eukaryotes
H3K4	SET1 SMYD	PfSET10	*Dm* Trx; *Hs* MLL1, MLL2, MLL3; *Hs* SET1; *Sc* SET1; *Hs* SET7/9; *Dm* ASH1
H3K9	SUV39	Unknown	*Dm* Su(var)3-9, *Hs* and *Mm* SUVAR39H1 and UVAR39H2; *Sp* CLR4, *Hs* and *Mm* G9a, Hs GLP1; *Hs* and *Mm* SETDB1, *Nc* DIM-5; *Dm* ASH1.
H3K36	SET2	PfSET2	*Sc* SET2; *Mm* NSD1
H3K27	EZ	Unknown	*Dm* Ez; *Hs* EZH1 and EZH1
H4K20	SUV4-20 SET8	TgSET8; PfSET8	*Hs* and *Dm* SET8; Dm, *Mm*, and *Hs* SUV4-20H1 and SUV4-20H2; *Mm* NSD1; *Dm* ASH1; *Sp* SET9.
H3K18	SMYD3	TaSETup1	Unknown

Species abbreviations: *Dm, Drosophila melanogaster, Hs, Homo sapiens; Mm, Mus musculus; Nc, Neurospora crassa; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe*; *Pf, Plasmodium falciparum; Tg, Toxoplasma gondii. Ta, Theileria annulata* Adapted from (Dillon SC et al. 2005)

Introduction

HKMTs are predicted to carry out gene regulation at two levels. The specific activation and repression of genes are termed "gene-level" regulation. And the methylation which would affect the organization of the basic structure of chromosomes is termed "chromosomal-level" regulation (Sims RJ III *et al,* 2003) (Figure 4).

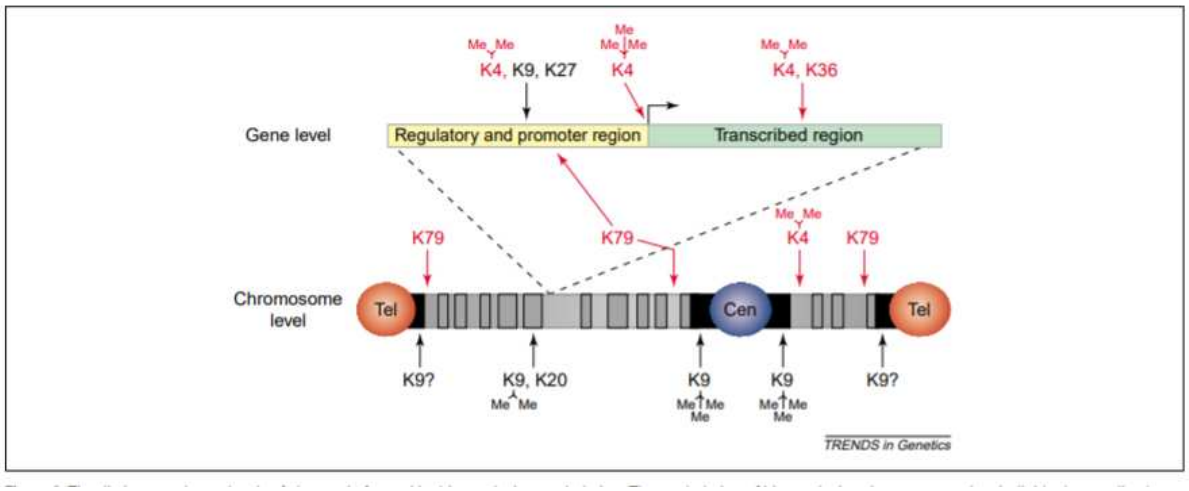


Figure 4. Histone lysine methylation events: gene (upper panel) and chromosome (lower panel) level. Methylation sites involved in gene activation and repression are represented in red and black respectively. Tel: Telomere; Cen: Centromere. Dark grey boxes represent heterochromatin. Light grey boxes represent euchromatin. Image adapted from (Sims RJ III et al. 2003).

Experiments using methods such as chromatin immunoprecipitation (ChIP) have advanced the understanding concerning the location of these methylation marks and in turn their role in gene expression regulation. Studies have shown that HKMTs mediate gene activation through methylation marks such as H3K4, H3K36, and H3K79. H3K4 methylation mark is always associated with transcriptionally active chromatin (Sims RJ III et al. 2003). Di-methylation of lysine 4 of histone 3 (H3K4Me2) and methylation of H3K79 are known to establish euchromatin regions which subsequently allow chromatin to be accessible by different HKMTs to perform gene-level regulation (Sims RJ III et al. 2003). Studies carried out in yeast have identified that H3K4 methylation catalyzed by SET1 accumulates near the 5'- mRNA coding region of genes and is associated with the early stages of transcription such as transcription activation (Santos-Rosa H *et al,* 2002). Whereas, SET2 in association with RNA polymerase II mediates H3K36 methylation driving transcription elongation (Li J *et al,* 2002).

HKMTs mediate gene repression through methylation marks such as H3K9, H3K27, and H4K20. Unlike gene activation marks, specific gene-level repression catalyzed by these marks

remains much less characterized. However, they are involved in the bulk silencing of chromatin. A certain degree of tri-methylation of lysine 9 of histone 3 (H3K9Me3) contributes to the highly condensed centromeric regions of chromosomes. H3K9 along with H3K27 methylation is known to participate in the X-chromosome inactivation process. H4K20 methylation is also responsible for silent chromatin. Thus, the major covalent histone modifications known to be associated with repression are observed to regulate at the chromosome level (Sims RJ III et al. 2003). Histone lysine methylations and their associated functions are summarized in Table 2.

Table 2 – Histone lysine methylations and their associated functions in Apicomplexa and other eukaryotes

Lysine methylation	**Apicomplexa**	**Function in other eukaryotes**
H3K4	*Tg* – Transcriptional activation *Pf* – Transcriptional activation	*Dm, Hs* - Transcriptional activation *Sc, Hs* - Transcriptional elongation
H3K36	*Pf* – Repression of transcription	*Sc* - Transcriptional elongation and silencing *Mm* – Transcriptional regulation
H3K79	Unknown	*Sc, Hs,* Demarcation of euchromatin
H3K9	*Tg* – Heterochromatin silencing *Pf* – Heterochromatin silencing; transcriptional repression	*Dm, Mm, Hs,* Heterochromatic and euchromatic silencing
H3K27	Unknown	*Dm, Hs, Mm* – Euchromatin silencing
H4K20	Tg – Heterochromatin assembly; cell cycle regulation. Pf – Heterochromatin assembly.	*Dm, Hs* - Cell cycle-dependent silencing, mitosis, and cytokinesis. *Dm, Mm, Hs* – Heterochromatin silencing

Species abbreviations: *Dm, Drosophila melanogaster, Hs, Homo sapiens; Mm, Mus musculus; Sc, Saccharomyces cerevisiae*; *Pf, Plasmodium falciparum; Tg, Toxoplasma gondii*. Adapted from (Dillon SC et al. 2005)

1.2.4. Histone proteins in Apicomplexa

Histone 3 (H3) and Histone 4 (H4) are the most evolutionarily conserved histones in Apicomplexa, perhaps due to the extensive post-translational modifications that can occur on these histones to affect gene expression. Based on phylogenetic analysis, most of the protozoan parasites have three H3 class histones: (i) canonical H3, (ii) H3.3, and (iii) CenH3. Canonical H3 and H3.3 are associated with transcriptionally active loci whereas CenH3 is present at the centromere to facilitate kinetochore formation (Sullivan WJ Jr et al. 2006). CenH3 is present in *P. falciparum* and *T. gondii* (Miao, J et al. 2006) (Brooks CF et al. 2011). However, gene prediction detected cgd4_2030 to represent CenH3 in *C. parvum*. Similar to other eukaryotes, Apicomplexa possess canonical H4 but no variant (Sullivan WJ Jr et al. 2006).

1.2.5. Histone lysine methylation machinery of Apicomplexa

The availability of genome sequences of *P. falciparum* (PlasmoDB), *T. gondii* (ToxoDB), and *C. parvum* (CryptoDB) has made it possible to identify putative homologs of the main families of histone-modifying enzymes (Sullivan WJ., Jr and Hakimi MA 2006). Even though, all the identified modifiers have not yet been characterized for their enzymatic activity.

Out of 14 predicted putative HKMTs in *T. gondii*, TgSET8 has been characterized as mono, di and tri methylate lysine 20 of histone 4 (H4K20Me1, H4K20Me2, H4K20Me3) (Sautel CF *et al,* 2007). Methylation caused by TgSET8 has been speculated to suppress tachyzoite gene expression and facilitate conversion to the bradyzoite stage. Whereas, *T. gondii*, TgKMTox, another lysine methyltransferase has been identified to regulate parasite's oxidative stress responses by upregulating the expression of anti-oxidant genes (Sautel CF *et al,* 2009). Similarly, TaSETup1 mono-methylated lysine 18 residue of histone 3 (H3K18Me1) controls stage differentiation in *Theileria annulata,* a bovine cell transforming parasite (Cheeseman, K *et al,* 2021). However, in *P. falciparum*, PfSET2/PfSETvs and PfSET10 are characterized HKMTs which are responsible for methylating H3K36 and H3K4 respectively and their activity regulates the expression of *var* genes responsible for virulence and immune evasion (Duffy MF *et al,* 2014) (Table 1) (Figure 5).

Histone methylation is a reversible event catalyzed by histone demethylases. Jumonji C-terminal (JmjC) domain-containing putative histone demethylases have been identified in *T. gondii, P. falciparum, Babesia bovis, T. annulata* (Cui L *et al,* 2008). Databases have also identified two lysine-specific demethylases (LSD) like proteins in the *T. gondii* genome (Saksouk N et al. 2005). However, to date, none of these putative demethylases have been enzymatically characterized.

1.2.6. Functional significance of histone lysine methylations in Apicomplexa

Apart from epigenetic modifications characterized by histone-modifying enzymes, advances have also been made in genome-wide mapping of multiple histone modifications. Techniques such as ChIP, have allowed the advancement of the research in Apicomplexa parasites which in general, be easily genetically manipulated. Moreover, the highly conserved N-terminal tails of histone 3 and histone 4 of Apicomplexan parasites make it easier to utilize an array of commercially available antibodies specific for different modifications (Sullivan WJ Jr et al. 2006).

As observed in other eukaryotes, the H3K4 methylation mark has also been known to occur at the promotor region of actively expressed genes in *T. gondii* (Gissot M and Kim K 2008). Whereas H3K9Me3 and H4K20Me3 were observed on repressed genes, especially in the heterochromatin domain (Sautel CF et al. 2007). However, mono-methylation of lysine 20 of H4K20Me1 was observed to peak during mitosis, thus indicating that it regulates the genes involved in parasite life cell cycle progression (Sautel CF et al. 2007). Even though not much is known about specific genes regulated by methylation marks in *T. gondii*, a significant process has been made on this front in *P. falciparum*, considering that much of the knowledge of epigenetic mechanism has been acquired through the study of *var* (antigenic variation) virulence genes. Tri-methylation of lysine 4 of histone 3 (H3K4Me3) modification in *P. falciparum* is significantly enriched in genes important for growth and interactions with the host environment (Duffy MF et al. 2014). The transcription start site of the transcribed *var* gene is enriched in H3K4Me3 during the immature ring stage and transiently repressed by H3K4Me2 as the parasite matures suggesting that lysine methylations are capable of conferring epigenetic memory for reactivation of the same *var* gene in the mitotic progeny (Lopez-Rubio JJ et al. 2007). Unlike, the known function of H3K36 methylation in other eukaryotes to be involved in gene activation (Sims RJ III et al. 2003), tri-methylation of lysine 36 of histone 3 (H3K36Me3) in *P. falciparum* has been shown to repress the expression of *var* genes. The

presence of H3K36Me3 at the 3' non-var genes in the ring stage and throughout the var genes in the schizont stage highlights the multifunctional role of this methylation in Apicomplexan parasites (Jiang L et al. 2013). *P. falciparum* lacks the H3K9Me3 mark at the pericentric heterochromatin that is required for kinetochore formation in other eukaryotes mainly indicating the chromosome level of regulation carried out by this methylation. But is involved at gene level regulation by inducing repression of var genes across their 5' UTRs and ORFs (Lopez-Rubio JJ et al, 2009). Unlike H3K9Me3, H4K20Me3 is associated with heterochromatin assembly and loss of this methylation mark does not affect var gene silencing (Sautel CF et al. 2007) (Lopez-Rubio JJ et al. 2009) (Table 2).

1.2.7. Effects of Apicomplexa infection on host epigenome

Understanding the stable changes brought about by epigenetic events in the regulation of gene expression in the host as a result of infection is of great relevance in the development of strategies to prevent the disease. Here we present, a brief overview of the effect of Apicomplexan parasites especially on the histone lysine methylation events in the host.

Effector proteins secreted by *T. gondii* induce gene repression in the infected host. *Toxoplasma* E2F4-associated EZH2-inducing Gene Regulator (TEEGR), the dense-granule effector, is exported into the host cell which eventually ends up in the nucleus and acts as a transcriptional activator of the epigenetic silencer EZH2, which controls the expression of a subset of pro-inflammatory cytokines to allow the persistence of parasite infection (Braun L et al. 2019). As explained previously, EZH2 is representative of a family of HKMTs, identified to methylate H3K27 and induce gene repression (Dillon SC et al. 2005). The TEEGR-EZH2 pathway leads to the repression of around 71 genes including a subset of NF-κβ- regulated cytokines such as IL-1β, Interleukin-6 (IL-6), Interleukin-8 (IL-8), and C-C motif chemokine ligand 20 (CCL20)) (Braun L et al. 2019).

On the other hand, one of the strategies employed by *T. annulata* includes induction of host matrix-metalloproteinases-9 (MMP-9) gene expression by activation marks such as H3K4Me3 on the MMP-9 promoter. Moreover, an analysis of a panel of mammalian KMTs revelated that *Theileria* infection led to elevated levels of SMYD3 methyltransferase. As explained previously, SMYD3 is a representative of a family of HKMTs, identified to methylate H3K4 and induce gene activation. As a result of *Theileria* infection, a SET domain containing protein, SMYD3 binds to DNA recognition motifs in the MMP9 promoter, leading to methylation of H3K4 and induction of MMP-9 expression (Cock-Rada AM et al. 2012). MMP-9, is well-

known to be associated with tumor metastasis and angiogenesis. Thus, strengthening one of the features of the cellular transformation induced by *Theileria* spp. of an invasive phenotype, similar to that observed in metastatic tumors (Tretina K et al. 2015). However, it is currently unclear as to how the parasite induces SMYD3 expression whether it is via parasite secreted effector proteins or through modulation of host signaling pathways.

Unlike *Theileria*, effector molecules in the form of non-coding RNA (ncRNA) are secreted from *Cryptosporidium* which is responsible for modulation of host gene expression. This ncRNA is reported to bind to G9a through a Ga-interacting protein, PRDM1. G9a is a histone methyltransferase known to methylate the histone at H3K9, a known repression mark (Dillon SC et al. 2005). Cdg7_FLc_0990, ncRNA, appears to be recruited to the promoter regions together with G9a for H3K9 methylation and subsequently resulting in transcriptional suppression of low-density lipoprotein receptor-related protein-5 (*LRP5*), Solute Carrier Family 7 Member 8 (*SLC7A8*), and Interleukin 33 (*IL33*) genes in the infected intestinal epithelium (Wang Y et al. 2017).

1.2.8. Epigenetic regulations in *Cryptosporidium*

Epigenetic regulations in *Cryptosporidium* have been studied to identify potential drug targets. In this respect, several commercially available drugs against an epigenetic modifier called histone deacetylases (HDACs) have been tested against *Cryptosporidium* infection (Darkin-Rattray S.J et al. 1996; Guo F et al. 2018a).

The identification of HDACs in *Cryptosporidium* was first encountered when Apicidin, a natural product, was shown to kill parasites by inhibiting HDACs. Concerning the mode of action, Apicidin seems to interfere with the process of acetylation-deacetylation of histones, eventually inducing hyperacetylation in the treated parasites (Darkin-Rattray S.J et al. 1996). The synergy between acetylation and deacetylation plays a significant role in transcriptional control in eukaryotic cells. Thus, targeting epigenetic modifiers became an attractive strategy to develop novel anti-parasitic agents. Interestingly, Vorinostat was identified to show inhibition against a specific class of *C. parvum* HDAC called CpHDAC3 (Guo F et al. 2018b). More recently, Nullscript was identified as an inhibitor of *Cryptosporidium* growth through inhibition of HDACs (Murakoshi F et al. 2020). However, which HDAC it targets is not clear.

Thus, CpHDAC3 an ankyrin repeats-containing, class II-2–like HDAC is the only epigenetic modifier that has been characterized by *C. parvum* (Rider SD Jr and Zhu G 2009). And as

previously described, *C. parvum* can modulate host gene expression by inducing methylation of H3K9.

In summary, we have given above some examples to demonstrate the role of epigenetics in the biology of apicomplexan parasites, and their interaction with their hosts. This overview is not exhaustive but illustrates the complexity of these mechanisms highlighting the urgency to explore this phenomenon, particularly in *Cryptosporidium* infection since there is a lack of knowledge concerning this parasite (Figure 5).

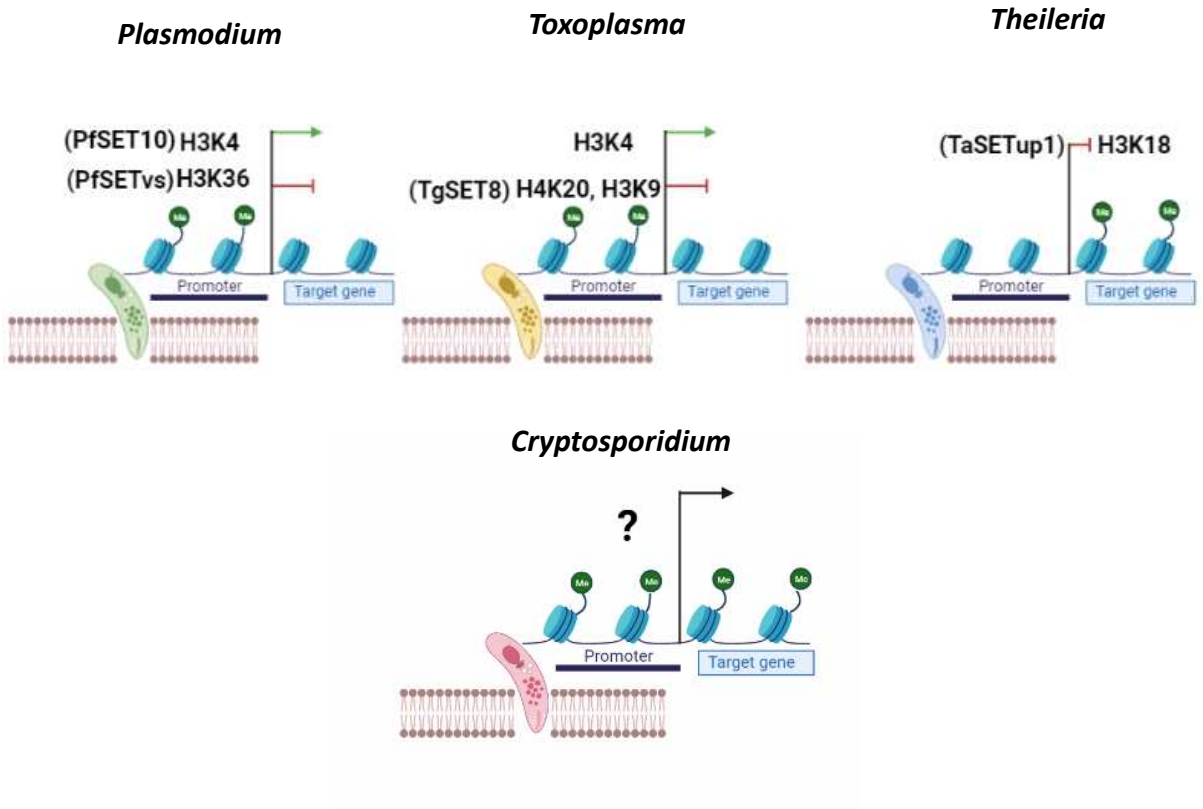


Figure 5. Schematic representation of histone lysine methylation events characterized in other Apicomplexa (*Plasmodium, Toxoplasma, and Theileria*) vs yet unexplored phenomenon in *Cryptosporidium*. In *Plasmodium*, HKMTs PfSET10 and PfSETvs methylate H3K4 and H3K36 respectively. In *Toxoplasma*, HKMT TgSET8 methylates H4K20. In *Theileria*, TaSETup1 methylates H3K18. Green arrow – gene activation, red arrow – gene repression.

1.3. *Cryptosporidium* and digestive cancer

Over 20% of cancers have been identified to be of infectious origins. The most common microbes such as viruses (*Human papillomavirus*, Epstein-Barr virus) and bacteria (*Helicobacter. pylori*). However, the contribution of eukaryotic intracellular parasites has been largely unexplored. Baring a few exceptions, such as *Theileria* and Cry*ptosporidium* which have been experimentally shown to induce host cellular transformation.

A very recent study about pathogens as risk factors for cancers in the gastrointestinal tract gives an updated overview of the epidemiological knowledge on the association between infections with bacteria and parasites and cancers of the gastrointestinal tract (Duijster JW et al. 2021). *Cryptosporidium* was one of the 13 pathogens to be investigated in association with gastrointestinal cancers. The study summarized that over threefold increased risk of *Cryptosporidium* infection was observed among cancer patients (all malignancies) as compared to non-cancer controls whereas for the digestive cancers an estimated risk of 3.7 fold is given for colorectal cancer in particular (Duijster JW et al. 2021).

In support of this ever-increasing association between *Cryptosporidium* and cancer, we summarized in our review an update on the latest literature available in the form of clinical, epidemiological, and experimental studies. Moreover, we speculated prospective mechanisms which might be employed by the parasite to induce cellular transformation.

Introduction

Cryptosporidium and colon cancer: cause or consequence?”

Manasi Sawant [1], Martha Baydoun [1,2], Colette Creusy [3], Magali Chabé [1,4], Eric Viscogliosi [1], Gabriela Certad [1,5,*], Sadia Benamrouz-Vanneste [1,6]

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d’Infection et d’Immunité de Lille, F-59000 Lille, France

[2] Université de Lille, Faculté des Sciences et Technologies, INSERM, CHU-Lille, U1189-ONCO-THAI-Assisted Laser Therapy and Immunotherapy for Oncology, F-59000 Lille, France.

[3] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59000 Lille, France.

[4] Faculté de Pharmacie, Univ. de Lille, F-59000 Lille, France.

[5] Délégation à la Recherche Clinique et à l’Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France.

[6] Equipe Ecologie et Biodiversité, Unité de Recherche Smart and Sustainable Cities, Faculté de Gestion, Economie et Sciences, Institut Catholique de Lille, F-59000 Lille, France.

* Correspondence: gabriela.certad@pasteur-lille.fr

Introduction

Abstract

The number of cancers attributable to infectious agents represents over 20% of the global cancer burden. The apicomplexan intracellular parasite *Cryptosporidium* is currently considered one of the major causes of mild and severe diarrhea worldwide. However, less attention has been paid to its tumorigenic potential despite the high exposure of humans and animals to this ubiquitous parasite. Herein, we discuss the potential causal link between *Cryptosporidium* infection and digestive cancer, with particular emphasis on colon cancer, based on increasing clinical, epidemiological, and experimental evidence supporting this association. In addition, we highlight the current knowledge about the potential mechanisms by which this parasite may contribute to cell transformation and parasite-induced cancer.

Keywords: *Cryptosporidium* infection, infection, and cancer, digestive cancer, colon cancer, carcinogenesis

Introduction

1. Introduction

One of the biggest obstacles to increasing life expectancy in the 21st century is cancer since this disease causes about 13% of human mortality [1]. Behind the strategy of treatment and early detection, it is important to work on cancer prevention, considering that between 40% and 45% of cancers are associated with preventable risk factors including tobacco smoke, lack of physical activity, obesity, dietary factors, exposition to solar ultraviolet (UV) radiation or infectious agents [2, 3]. Subsequently, reducing the burden of cancer is possible if these risk factors could be identified and if population exposure to them could be avoided or at least reduced [3]. Particularly, the role of some infectious agents as carcinogens has been already recognized by the International Agency for Research on Cancer (IARC). However, additional pathogens are probably involved in specific human cancers. This review will thus be focused on the causal link between infection and cancer, including an update on the association between the infection by the protozoan *Cryptosporidium* and digestive cancer. Several experimental and epidemiological studies have revealed this link further strengthened by mechanistic studies that show that this parasite can hijack the host cell machinery potentially leading to a transformation of the host cell.

2. Infection, an important cause of cancer

Causal associations between infectious agents and the development of human cancers have already been highlighted [4]. Overall, the total number of cancer attributable to infectious agents in 2002 was estimated at 1.9 million cases, standing for 17.8% of the global cancer burden. Moreover, it has been hypothesized that by 2050, the majority of human cancers could be due to infections [5,6]. However, proving that infectious agents are causative factors of human cancer remains difficult for many reasons as:

- The periods between primary infection and malignant transformation are frequently very long [7]
- Even if the majority of the infectious agents associated with human cancers are ubiquitous and common in the human population, only a small proportion of infected individuals develops cancer
- Some infections are linked to cancer development as associated risk factors [8]

Introduction

- Infectious agents act mainly as indirect oncogenes, without persistence of their genes within the respective cancer host cells. The most common indirect infectious carcinogens are agents causing immunosuppression as Human Immunodeficiency Virus (HIV) leading to Kaposi's sarcoma, or inflammation such as the bacteria *Helicobacter pylori*, the trematode *Schistosoma hematobium* and the Hepatitis C and B viruses [7]
- The main mechanisms by which infectious agents promote cancer are not necessarily involving direct mutagenesis, but instead are due to the complex interactions between hosts and pathogens [8]
- An infectious agent may trigger the initial events of oncogenesis while being absent in the final tumor [7]
- Pathogens associated with cancer are directing pathogen-driven processes leading to cell transformation. However, many non-oncogenic pathogens can also regulate these processes, indicating that other factors must be involved [8]
- In the cases of viruses, oncogenesis can occur through the persistence of the viral genome in a latent form in an infected host cell, either without replication or through the integration of the viral genome into a host-cell chromosome [8].
- Koch's postulates for proving a causal connection between a particular infectious agent and a disease cannot be applied to many human diseases as it would be unethical to experimentally infect humans with a potentially lethal infectious agent [8]
- Existing diagnostic tools may not be sensitive enough to link infectious agents with cancer development or testing may occur too long after the exposure [9].

Nevertheless, at least 11 biological agents have presently been recognized by the IARC as major contributors to the global number of cancers in humans (Figure 1).

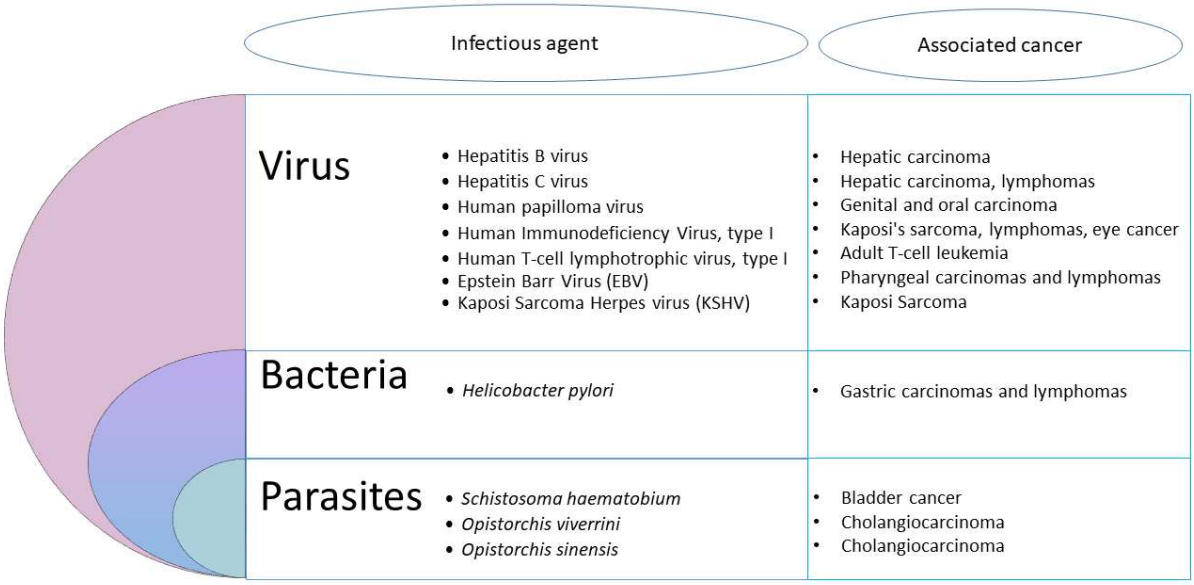


Figure 1. Infectious agents recognized by the International Agency for Research on Cancer (IARC) as major contributors to the global number of cancers.

These agents include viruses, bacteria and helminths. The most important infectious agents worldwide are *H. pylori* (5.5 % of all cancer), the human papilloma viruses (HPVs) (5.2 %), the hepatitis B and C viruses (4.9 %), the Epstein-Barr virus (EBV) (1%), the HIV together with the human herpes virus 8 (0.9%) and the Human T cell Leukemia/lymphoma *Virus* type 1 (HTLV-I) (0.03%) [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)]. Other pathogens, including parasites, are also considered as carcinogenic agents in human beings. Among helminths, the widespread digenetic trematode *S. haematobium* has been associated with urinary bladder cancer, and the flukes *Opisthorchis viverrini* and *Clonorchis sinensis* are causally linked with cholangiocarcinoma [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)]. So the idea of parasites as the cause of cancer in vertebrates is slowly developing [11]. However, the contribution of intracellular eukaryotic parasites to cancer development has been largely neglected until now [2]. Yet based on clinical and epidemiological evidences, many reports underlined a potential association between parasitic protozoan infections and cancer. Hence, the flagellate *Trichomonas vaginalis* was suspected to be associated with prostate [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)] and cervical cancers [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)], while the apicomplexan *Toxoplasma gondii* was suggested to be linked with ocular tumor,

meningioma, leukemia and lymphomas [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)]. It was also suggested that *Plasmodium falciparum* could play a cofactor role in the development of Burkitt lymphoma [14]. Nevertheless, only the apicomplexan genera *Cryptosporidium* and *Theileria* have been shown to induce cell transformation experimentally [2].

Pathogens use several strategies to target cellular processes during their parasitic interactions with the host cell. The identification of microbial proteins manipulating host functions to promote infection, proliferation and escape defenses has led to great progress in understanding the host's cellular processes. Continued persistent infection by a pathogen requires host-cell survival, host-cell proliferation, and evasion of the immune system by the pathogen. These pathogen-driven processes are achieved through various mechanisms that interfere with normal cell physiology. Alterations in these normally highly regulated pathways can lead to transforming events that have been described as the 'hallmarks of cancer' [5]. Carcinogenic pathogens are also able to target epigenetic mechanisms to divert the host cellular machinery [2]. Interestingly, recent mechanistic studies suggest that apicomplexan eukaryotic intracellular parasites are indeed capable of reproducing some mechanistic aspects of tumorigenesis leading to cancers, either by their infection alone, or by the combination of the parasitic infection with environmental factors [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)]. There is a growing number of pharmacological studies analyzing the effect of anti-cancer molecules on parasitic diseases and *vice versa* [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)]. Overall, the infection seems to play a crucial role in the etiology of cancer. Actually, it was estimated that there would be 26.3% fewer cancers in developing countries and 7.7% in developed countries if cancers associated with infectious diseases were prevented [(IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans 2012)].

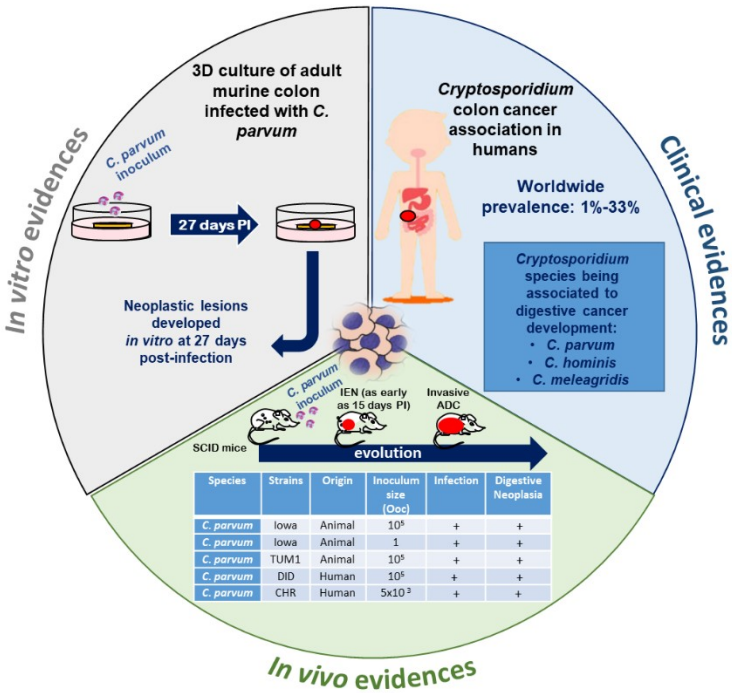
3. The special case of *Cryptosporidium*: a public health issue

Cryptosporidium is the agent of cryptosporidiosis, an infection resulting from ingestion of parasite oocysts mainly through the consumption of fecal contaminated food or water, or through direct contact with the infected host [19,20]. *Cryptosporidium parvum* and *C. hominis* are the two species responsible for the majority of human cases of cryptosporidiosis. This parasite is considered a major cause of diarrhea worldwide. It causes self-limited watery

diarrhea in immunocompetent individuals, but has devastating effects in those who are immunocompromised. In young children, malnutrition, growth and cognitive deficits were reported as sequels of cryptosporidiosis [21,22]. Most strikingly, a cohort study (GEMS) involving 22,500 children in Africa and Asia revealed that *Cryptosporidium* is one of the four main pathogens responsible for severe diarrhea and mortality in infants and toddlers [23]. This parasite was then considered as the second leading cause of death in children due to diarrhea [24]. More recently, the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) analyzed in 2016 the burden of diarrhea in 195 countries and reported that *Cryptosporidium* is the fourth leading cause of diarrhea mortality among children under 5 years of age (with 48301 annual death) [25,26]. It has also been reported that the substantial short-term burden of diarrhea induced by *Cryptosporidium* infection on childhood growth and well-being is largely underestimated [25,27] probably due to a significant proportion of asymptomatic or mild and self-limiting infections, that remain consequently not diagnosed. In addition, *Cryptosporidium* species are responsible for numerous waterborne outbreaks of gastrointestinal disease. The most extensive was described in 1993 in Milwaukee, USA, where over 400,000 people became ill (the population of this area was approximately 1.61 million) with 69 deaths [28,29]. The number of outbreaks caused by *Cryptosporidium* is increasing worldwide since 239 waterborne outbreaks were reported in Europe, Australia and North America between 2011 and 2016 *versus* only 120 in the same area between 2004 and 2010 [30,31]. The Centers for Disease Control and Prevention (CDC) also published that 32 outbreaks were caused by *Cryptosporidium* in the United States in 2016 and linked them to swimming pools and water playgrounds against only 13 in 2013 [32]. As a result, an ever-growing number of people would be exposed to this pathogen and not only in developing countries. Despite its prevalence and impact on public health, neither treatment nor vaccine against *Cryptosporidium* are yet available.

4. *Cryptosporidium* and cancer: a growing number of evidences

In various animal groups and humans, epidemiological and experimental studies tend to reinforce the hypothesis of an association between *Cryptosporidium* infection and cancer (Figure 2).



Species	Strains	Origin	Inoculum size (Ooc)	Infection	Digestive Neoplasia
C. parvum	Iowa	Animal	10^5	+	+
C. parvum	Iowa	Animal	1	+	+
C. parvum	TUM1	Animal	10^5	+	+
C. parvum	DID	Human	10^6	+	+
C. parvum	CHR	Human	$5x10^3$	+	+

Figure 2. Recent experimental and clinical approaches contribute to expanding the understanding of the role of *Cryptosporidium* in the induction of digestive neoplasia. PI: post-infection, IEN: intraepithelial neoplasia, ADC: adenocarcinoma, Ooc: oocysts. Source of pictograms: https://fr.freepik.com/photos-vecteurs-libre/banner">Banner vecteur créé par pch.vector - fr.freepik.com.

4.1 Clinical studies in humans

Several growing clinical evidences about links between cryptosporidiosis and human digestive neoplasia in different populations strengthen the idea of a possible causal relationship. The association of cryptosporidiosis and colonic adenocarcinoma was evoked in the case of a Spanish patient presenting both, who died quickly after the onset of clinical manifestations [33]. A cryptosporidiosis case of the biliary tract clinically mimicking a pancreatic cancer in an HIV/Acquired Immunodeficiency Syndrom (AIDS) patient was also described [34]. Other studies reported elevated colon carcinoma risk in AIDS patients with cryptosporidiosis [35] and bile duct carcinoma associated with *Cryptosporidium* infection in children with X-linked hyper-IgM syndrome in immunodeficient mice [36-38]. Authors of the latter studies proposed

that the mutation responsible for this defect might favor the colonization of the biliary epithelium by *Cryptosporidium*. Chronic infection by this parasite will follow and inflammation may be the cause of the malignant transformation [37,38]. Even if this condition is mainly diagnosed in children, a case of an adult patient with CD40L deficiency who suffered from cholangiocarcinoma arising from sclerosing cholangitis associated with chronic cryptosporidiosis was also reported [39]. In parallel, a clinical study conducted in Lebanon strongly suggested evidence for a link between cryptosporidiosis and colorectal cancer. Indeed, *Cryptosporidium* infection was identified by PCR in 21% (15/72) of biopsies from patients with recently diagnosed colon neoplasia (including low- or high-grade intraepithelial neoplasia and not invasive/invasive adenocarcinoma) before any treatment compared to 7% (9/146) of biopsies from patients without digestive neoplasia but with persistent gastrointestinal symptoms. The risk of *Cryptosporidium* infection was thus 4 times higher in the first group. Moreover, the molecular characterization of the corresponding *Cryptosporidium* isolates allowed the identification of either *C. parvum* or *C. hominis*. In addition, the presence of *Cryptosporidium* developmental stages in the apical position within the epithelial cells of the intestinal glands was confirmed [40] (Figure 3).

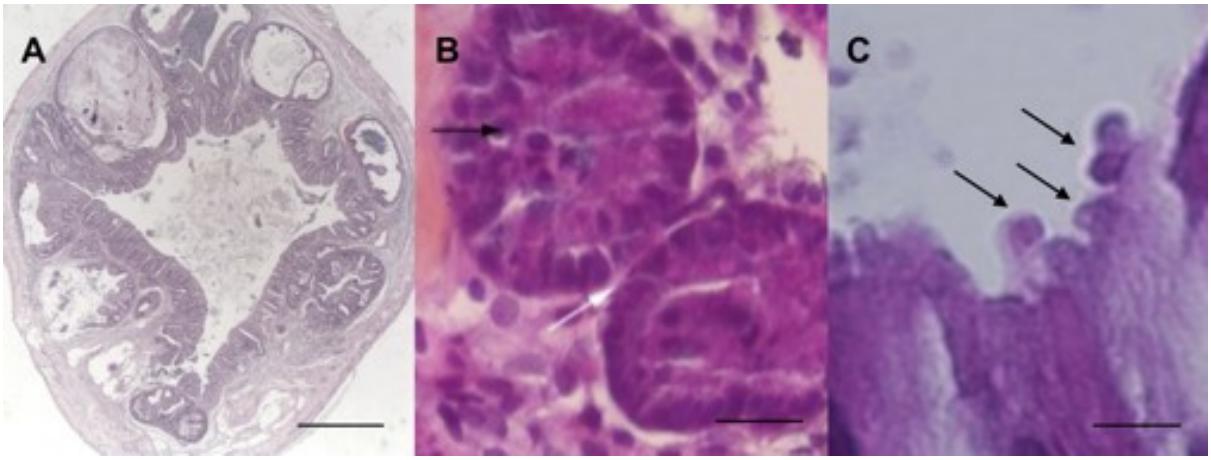


Figure 3. (A) Adenocarcinoma of the ileo-caecal region in a Dex-treated SCID mouse after 62 days of *C. parvum* infection. Scale bar, 1,000 µm. (B) Low-grade intraepithelial neoplasia in a murine colonic explant after 27 days of *C. parvum* infection characterized by reduction of the interglandular space (white arrow), and loss of nuclear polarity with slight pseudostratification (black arrow). Scale bar, 25 µm. (C) *Cryptosporidium* developmental stages were observed in apical position (arrows) within the epithelial cells in a human colon adenocarcinoma of a Lebanese patient. Scale bar, 5 µm. (Hematoxyline & Eosine).

Consistently, various clinical studies conducted in Poland confirm these observations [41-43]. On the other hand, in another cohort of immunocompetent patients from Poland with colorectal cancer, the presence of *Cryptosporidium* was found in one patient out of 145. After genotyping, the presence of *C. meleagridis* was identified, being this the first study reporting association of this *Cryptosporidium* species with colon adenocarcinoma [44]. More recently, a Chinese case-control study described a *Cryptosporidium* infection rate of 17.24% (20/116) in patients with colorectal cancer before chemotherapy. In addition, the same authors reported the presence of the parasite in liver, esophageal and small intestine cancers [45]. A summary of different reports about the link between *Cryptosporidium* infection and human digestive neoplasia in different populations is shown in Table 1.

Table 1. Associations between *Cryptosporidium* infection and human digestive neoplasia in different populations,

Type of cancer	Type of study	Geographic localization	Clinical sample: laboratory method	N (%)	P-value	Immuno-suppression	Reference
Colonic adenocarcinoma	Case report	Spain	Not reported	1	NA[a]	No	[33]
Pancreatic cancer	Case report	Brazil	Tissues /Microscopical observation	1	NA[a]	HIV/AIDS	
Colorectal cancer (Adenocarcinoma)	Data matching between HIV/AIDS and cancer registry databases in 16 U.S. states	United States	Tissues /Microscopical observation	3/269 (1%)	0.70	HIV/AIDS	[35]
Colorectal squamous cell carcinoma	Data matching between HIV/AIDS and cancer registry databases in 16 U.S. states	United States	Tissues /Microscopical observation	1/8 (12.5%)	0.02[b]	HIV/AIDS	[35]
Uncommon (colorectal cancers)	Data matching between HIV/AIDS and cancer registry databases in 16 U.S. states	United States	Tissues /Microscopical observation	3/43 (7%)	0.04[b]	HIV/AIDS	[35]

Bile duct carcinoma	Case reports	United States	Tissues /Microscopical observation	Not reported	NA[a]	X linked immunodeficiency with hyper-lgM	[36]
Hepatoma	Analysis of the USIDNET Registry	United States	Not reported	1/145 (1%)	NA[a]	X-linked hyper-IgM syndrome in children	[38]
Cholangiocarcinoma	Case report	United Kingdom	Stool samples, Coprological analysis	1	NA[a]	CD40L deficiency	[39]
Colonic adenocarcinoma	Case-control	Lebanon	DNA from biopsies, PCR	15/72 (21%)	0.003[b]	No	[41]
Colonic adenocarcinoma	Cases	Poland	Stool samples, coprology and ELISA	4/55 (18%)	NA[a]	No	[42]
Colonic adenocarcinoma	Cases	Poland	Stool samples, ELISA	10/87 (12%)	NA[a]	No	[43]
Colonic adenocarcinoma	Case-control	Poland	Stool samples, coprology analysis and ELISA	14/108[b] (13%)	0.015[b]	No	[44]
Colonic adenocarcinoma	Cases	Poland	DNA from stools, PCR	1/145 (1%)	NA[a]	No	[45]
Colonic adenocarcinoma	Case-control	China	DNA from stools, PCR	20/116 (17.24%)	<0.001 [b]	No	[45]
Gastric	Case-control	China	DNA from stools, PCR	2/51 (4%)	0.121	No	[45]
Esophageal	Case-control	China	DNA from stools, PCR	1/16 (6.25%)	0.029 [b]	No	[45]
Liver	Case-control	China	DNA from stools, PCR	1/7 (14.29%)	<0.001 [b]	No	[45
Small Intestine	Case-control	China	DNA from stools, PCR	2/5 (40%)	<0.001 [b]	NO	[45]

[a] NA, Not applicable. [b] The difference between the prevalence of the cases and controls is statistically significant

Besides direct clinical evidence suggesting the association between *Cryptosporidium* and digestive cancer development, some studies conveyed that the risk of developing colon carcinoma is significantly elevated among AIDS patients, a group at risk for *Cryptosporidium* infection [46]. However, *Cryptosporidium* is an opportunistic agent that causes important morbidity and mortality in persons with immunodeficiency. Therefore, it is possible that immunocompromised people have a higher risk of developing malignancy induced by this parasite, especially when their immunosuppression is more severe. In fact, *Cryptosporidium* infection has been suggested to be associated with some malignancies such as leukemia [47]. Nevertheless, a meta-analysis recently reported that, even if a positive association was found between *Cryptosporidium* infection and cancer in general (OR=3.3; 95 CI: 2.18-4.98), the occurrence of *Cryptosporidium* infection was mainly related to colorectal cancer (OR=3.7; 95 CI: 2.10-6.50) but not to other types of malignancies, such as blood cancer [48], suggesting

that the parasite is not a special risk to cancer patients. Even if there are several clinical evidence from different geographical areas, the majority of the articles in the literature are case reports. Further prospective studies should be conducted based on clinical trials using sensitive diagnostic tools for the identification of the parasite.

4.2. Natural or experimental infection in animals

Links between non-malignant tumors or atypical histology and *Cryptosporidium* were already reported in naturally or experimentally infected animals. For example, two studies described an association between aural or aural-pharyngeal polyps and *Cryptosporidium* infection in iguanas [49,50]. Intestinal metaplasia of the proventriculus was also associated with *Cryptosporidium baileyi* in a snowy owl [51]. Moreover, histological analysis of twenty three leopard geckos revealed a *Cryptosporidium* sp. infection associated with hyperplasia of small and large intestine [52]. To complete this overview, low grade dysplasia in bile ducts has also been reported in an experimental model of IFN-δ knockout mice infected with *C. parvum* [37]. Nevertheless, no studies to our knowledge, described an association between *Cryptosporidium* sp. infection and malignant tumors, until 2007 [53]. Indeed, to explore the dynamics of *Cryptosporidium* infection, an animal model of cryptosporidiosis was developed using corticoid dexamethasone-treated or untreated adult Severe Combined Immunodeficiency (SCID) mice, orally infected with *C. parvum* or *C. muris* oocysts. Intriguingly, only *C. parvum*-infected animals developed ileo-caecal adenocarcinoma as soon as 45 days post-infection even when mice were infected with only one oocyst (Figure 3) [53,54]. The inoculation of animals was performed with the *C. parvum* IOWA strain isolated from cattle and which is the reference strain for this *Cryptosporidium* species (first *C. parvum* strain to have an entire genome sequenced). However, additional *C. parvum* strains were tested in the same murine model including “TUM1” (isolated from a calf in the USA), and “Did” and “CHR” (isolated from patients in a Lille hospital, France). These three isolates were found to be more virulent than the IOWA strain [55-57]. Indeed, they induced a higher mortality rate, an earlier onset of neoplastic lesions (as soon as 15 days post-infection for the CHR strain), and a more rapid progression to invasive adenocarcinoma. Interestingly, the development of intestinal low and high grade dysplasia after only 30 days post-infection with *C. parvum* in a model of dexamethasone treated immunocompetent Swiss albino mice was reported by others [58]. However, adenocarcinoma development was not detected in this model, probably due to the

early time of euthanasia. Similar observations were reported by others using the same mice model [59] [60].

4.3. *In vitro* models

Recently, a three-dimensional (3D) *in vivo* like culture model from adult murine colon was developed allowing biological investigations of *Cryptosporidium* infection and a better study of its pathophysiology. Indeed, the resulting system allowed the maintenance of the infection but also the development of low grade intra-epithelial neoplasia *in vitro* after only 27 days post-infection (Figure 3) [61]. This model is currently adapted to human intestinal tissue to look for further evidences on the role of *C. parvum* and/or *C. hominis* in the induction of human colon cancer.

5. Hypotheses about molecules and mechanisms involved in the induction of tumorigenesis by *Cryptosporidium*

The pathophysiological mechanisms of *Cryptosporidium* infection are multifactorial and not completely understood. Some advances were achieved recently and revealed that the infection by *C. parvum* induces cytoskeleton remodeling and actin reorganization through the implication of several intracellular signals involving for example, PI3K, Src, Cdc42 and GTPase [62,63] (Figure 4).

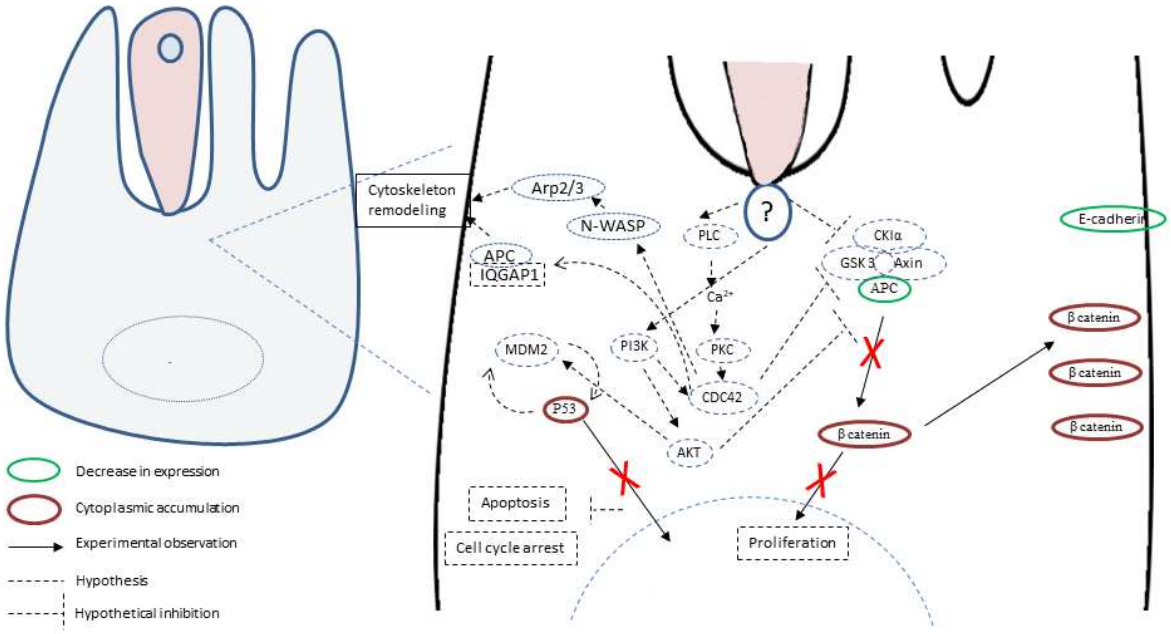


Figure 4. Hypothetical host cell signaling pathways linking *Cryptosporidium parvum* infection and tumorigenesis. Different studies have shown that *C. parvum* induces cytoskeleton remodeling and actin reorganization through the implication of several intracellular signals including phosphatidylinositol 3-kinase (PI3K) and Cdc42. In addition, in an experimental model, Adenomatous Polyposis Coli (APC) and E-cadherin labeling were decreased while β-catenin and the P53 tumor suppressor labeling were increased in the cytoplasm of *C. parvum* infected epithelial cells. β-catenin was found localized in a juxtamembraneous position, suggesting a role of the non-canonical Wnt pathway in this transformation process. P53 was not translocated into the nucleus and its labeling increased in the cytoplasm where P53 could regulate key metabolic pathways associated to apoptosis and cell cycle arrest.

Consistently, signal transduction pathways targeting cell proliferation, cellular junctions and adhesions have also been described in gastric cancer induced by *H. pylori* [64].It was also reported that the infection by *C. parvum* leads to the activation of NF-κB [62], known to induce anti-apoptotic mechanisms and also transmit oncogenic signals to epithelial cells [65,66]. In addition, microarray assays were recently performed on *C. parvum* IId Human Ileocecal Adenocarcinoma (HCT-8) infected cells. A differential profile of mRNAs was found between infected and non-infected cells. Indeed, mRNAs of the Wnt and hedgehog signaling pathways were significantly differentially expressed in infected cells compared to not infected ones [67].

Noticeably, these two pathways are also involved in the development and progression of colorectal cancer [68]. Despite the growing evidence about the hijacking of cellular pathways maybe involved in cancer onset, this information has rarely been linked to the tumorigenic potential of the parasite. To our knowledge, only one study tried to decipher this process and highlighted the important role of the Wnt signaling pathway and the alteration of the cytoskeleton in the carcinogenic process induced by *C. parvum* experimental infection [69]. Indeed, the immunohistochemical analysis of ileocecal region sections embedded in paraffin from *C. parvum* infected *versus* non-infected mice showed alterations in APC, β-catenin, P53 and E-cadherin expression [69]. APC and E-cadherin labelings were decreased while those of β-catenin and P53 were increased in the cytoplasm of epithelial cells. In addition, the immunofluorescence analysis of these histological sections confirmed a membranous and juxtamembraneous localization of β-catenin without nucleus translocation, suggesting an involvement of the non-canonical Wnt pathway [69]. But, unlike *Helicobacter pylori*, for which bacterial virulence factors associated to the gastric cancer outcome were identified, such as CagA, VacA and OipA [70], virulence factors implied in the *C. parvum* induced carcinogenic process remains yet unknown. The whole genome sequences of different species and isolates of *Cryptosporidium* are now available [71-73]. Hence, a comparative genomic analysis of different *C. parvum* strains (Did, TUM1, CHR and IOWA, the reference strain) with variable virulence was recently performed [57]. Overall, 125 common SNVs corresponding to 90 CDSs were found in the three more virulent strains (Did, TUM1 and CHR) compared to IOWA strain. The majority of these genes are over-expressed in the intracellular stages of the parasite. Behind the different identified mutated genes, mucins, transporters (ABC and ATPase3) and cysteine proteases were also found, being these genes already described as virulence factors. This study also reported new potential factors involved in the virulence of *C. parvum* such as various phosphatases (PP2A, Cdc14) and a histone-lysine N-methyltransferase. Further investigations are needed to elucidate carcinogenic mechanisms induced by *C. parvum*. In particular, the biological function study of the potential virulent and/or carcinogenic factors identified could be facilitated by the use of genome editing tools like CRISPR/Cas9 [57].

Conclusions and future directions

Is colon cancer a cause or a consequence of *Cyptosporidium* infection? We presented an updated picture of the link between digestive cancer and infection by this parasite. Available experimental and clinical data synthesized herein suggest that the parasite is able to employ

strategies to target cellular processes during its complex interactions with host cells leading to a parasite-induced transformation. Despite several evidences about associations between cryptosporidiosis and digestive neoplasia, it seems that not enough attention has been paid to the tumorigenic power of this protozoan parasite. The fact that *Cryptosporidium* is an opportunistic agent is the main difficulty for proving its role in the induction of human digestive cancers. Therefore, it is necessary to demonstrate a direct causal link, and to identify virulent factors and carcinogenic mechanisms responsible for the epithelial cell transformation. A potential solution would be to combine recent advances in 3D culture models, comparative genomic studies and transfection methods (CRISPR/Cas9 system) together with further clinical trials using sensitive diagnostic tools. If the causal link between *Cryptosporidium* and human cancer is clearly established, a great number of digestive cancers could be prevented using public health measures to reduce the risk of *Cryptosporidium* infection. In addition, this could incite researchers to explore new therapeutic targets and vaccines in order to clear or prevent the infection and *in fine* to save hundreds of thousand children from severe diarrhoea and mortality [24]. Research into this topic is urgently needed, since the incidence of *Cryptosporidium* infection is increasing worldwide.

Search strategy and selection criteria

We selected articles from PubMed and Google Scholar using the research terms "*Cryptosporidium*", "cancer", "oncogenesis", "infection and cancer", "colorectal cancer", "cryptosporidiosis", "parasites and cancer", "*Cryptosporidium* and cancer", "cancer causes", "epigenetics and cancer", "*Cryptosporidium* and virulence factors", "digestive neoplasia", "colon adenocarcinoma", "cancer and mechanisms". Reviews, clinical, epidemiological and experimental data were taking into account without restriction of date or language. Preference was given to the articles published within the past 20 years.

Author contributions: Conceptualization, M.S., E.V., G.C., and S.B.-V.; Formal analysis, M.S., M.B., C.C., M.C., E.V., G.C., and S.B.-V.; Funding acquisition, E.V., G.C., and S.B.-V.; Resources, C.C., G.C. and S.B.-V.; Writing-original draft, M.S., E.V., G.C., and S.B.-V.; Writing-review and editing, M.S., M.B., C.C., M.C., E.V., G.C., and S.B.-V.

Funding: This research was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Institut Pasteur of Lille, the University of Lille, the University Catholic of Lille, the CHRU of Lille and the Plan Cancer

"Epigenetics and cancer 2015" (PARACAN, EPIG2015069). M.S. was supported by a PhD fellowship from the University of Lille.

Conflicts of interests: The authors declare no conflict of interest.

References

1 Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424.

2 Cheeseman, K.; Certad, G.; Weitzman, J.B. Parasites et cancer : existe-t-il un lien ? *Médecine/Sciences* **2016**, *32*, 867–873.

3 Whiteman, D.C.; Wilson, L.F. The fractions of cancer attributable to modifiable factors: A global review. *Cancer Epidemiol*. **2016**, *44*, 203–221.

4 Benamrouz, S.; Conseil, V.; Creusy, C.; Calderon, E.; Dei-Cas, E.; Certad, G. Parasites and malignancies, a review, with emphasis on digestive cancer induced by *Cryptosporidium parvum* (Alveolata: Apicomplexa). *Parasite* **2012**, *19*, 101–115.

5 Bañuls, A.L.; Thomas, F.; Renaud, F. Of parasites and men. *Infect. Genet. Evol.* **2013**, *20*, 61–70.

6 Bouvard, V.; Baan, R.; Straif, K.; Grosse, Y.; Secretan, B.; El Ghissassi, F.; Benbrahim-Tallaa, L.; Guha, N.; Freeman, C.; Galichet, L.; et al. A review of human carcinogens - Part B: biological agents. *Lancet Oncol.* **2009**, *10*, 321-322.

7 Zur Hausen, H. The search for infectious causes of human cancers: where and why. *Virology* **2009**, *392*, 1–10.

8 Dalton-Griffin, L.; Kellam, P. Infectious causes of cancer and their detection. *J Biol* **2009**, *8*, 67.

9 O'Connor, S.M.; Taylor, C.E.; Hughes, J.M. Emerging Infectious Determinants of Chronic Diseases. *Emerg. Infect. Dis.* **2006**, *12*, 1051–1057.

10 IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans. Biological Agents, volume 100 B, a review of human carcinogens. Lyon, France, 2012

http://monographs.iarc.fr.

11 Ewald, P.W.; Swain Ewald, H.A. Infection and cancer in multicellular organisms. *Philos. Trans. R. Soc. B Biol. Sci.* **2015**, *370*, 20140224.

12 Stark, J.R.; Judson, G.; Alderete, J.F.; Mundodi, V.; Kucknoor, A.S.; Giovannucci, E.L.; Platz, E.A.; Sutcliffe, S.; Fall, K.; Kurth, T.; et al. Prospective study of *Trichomonas vaginalis* infection and prostate cancer incidence and mortality: Physicians' Health Study. *J. Natl. Cancer Inst.* **2009**, *101*, 1406–1411.

13 Yang, S.; Zhao, W.; Wang, H.; Wang, Y.; Li, J.; Wu, X. *Trichomonas vaginalis* infection-associated risk of cervical cancer: A meta-analysis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2018**, *228*, 166–173.

14 Khurana, S.; Dubey, M.L.; Malla, N. Association of parasitic infections and cancers. *Indian J. Med. Microbiol.* **2005**, *23*, 74–79.

15 Marsolier, J.; Perichon, M.; DeBarry, J.D.; Villoutreix, B.O.; Chluba, J.; Lopez, T.; Garrido, C.; Zhou, X. Z.; Lu, K. P.; Fritsch, L.; et al. *Theileria* parasites secrete a prolyl isomerase to maintain host leukocyte transformation. *Nature* **2015**, *520*, 378–382.

16 Robbiani, D.F.; Deroubaix, S.; Feldhahn, N.; Oliveira, T.Y.; Callen, E.; Wang, Q.; Jankovic, M.; Silva, I.T.; Rommel, P.C.; Bosque, D.; et al. *Plasmodium* infection promotes genomic instability and AID-dependent B cell lymphoma. *Cell* **2015**, *162*, 727–737.

17 Checkley, W.; White, A.C.; Jaganath, D.; Arrowood, M.J., Chalmers, R.M., Chen, X.M., Fayer, R., Griffiths, J.K.; Guerrant, R.L.; Hedstrom, L.; et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *Lancet Infect. Dis.* **2015**, *15*, 85–94.

18 Plummer, M.; de Martel, C.; Vignat, J.; Ferlay, J.; Bray, F.; Franceschi, S. Global burden of cancers attributable to infections in 2012: a synthetic analysis. *Lancet Glob. Health* **2016**, *4*, e609–16.

19 Putignani, L.; Menichella, D. Global distribution, public health and clinical impact of the protozoan pathogen *Cryptosporidium*. *Interdiscip. Perspect. Infect. Dis.* **2010**, *2010*, 753512.

20 Ryan, U.; Fayer, R.; Xiao, L. *Cryptosporidium* species in humans and animals: current

understanding and research needs. *Parasitology* **2014**, *141*, 1667–1685.

21 Bhalchandra, S.; Cardenas, D.; Ward, H.D. Recent breAKThroughs and ongoing limitations in *Cryptosporidium* research. *F1000Res.* **2018**, *7*, F1000 Faculty Rev-1380.

22 Lilja, M.; Widerström, M.; Lindh, J. Persisting post-infection symptoms 2 years after a large waterborne outbreak of *Cryptosporidium hominis* in northern Sweden. *BMC Res. Notes* **2018**, *11*, 625.

23 Kotloff, K.L.; Nataro, J.P.; Blackwelder, W.C.; *et al.* Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet* **2013**, *382*, 209–222.

24 Gibson, A.R.; Striepen, B. *Cryptosporidium*. *Curr. Biol.* **2018**, *28*, R193–194.

25 Khalil, I.A.; Troeger, C.; Rao, P.C.; Blacker, B.F.; Brown, A.; Brewer, T. G.; Colombara, D.V.; De Hostos, E. L.; Engmann, C.; Guerrant, R. L.; et al. Morbidity, mortality, and long-term consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyzes study. *Lancet Glob. Health* **2018**, *6*, e758–768.

26 GBD 2016 Diarrhoeal Disease Collaborators. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect. Dis.* **2018**, *18*, 1211–1228.

27 Widerström, M.; Schönning, C.; Lilja, M.; Lebbad, M.; Ljung, T.; Allestam, G.; Ferm, M.; Björkholm, B.; Hansen, A.; Hiltula, J.; et al. Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. *Emerg. Infect. Dis.* **2014**, *20*, 581–589.

28 Corso, P.S.; Kramer, M.H.; Blair, K.A.; Addiss, D.G.; Davis, J.P.; Haddix, A.C. Cost of illness in the 1993 waterborne *Cryptosporidium* outbreak, Milwaukee, Wisconsin. *Emerg. Infect. Dis.* **2003**, *9*, 426–431.

29 Mac Kenzie, W.R.; Hoxie, N.J.; Proctor, M.E.; Gradus, M.S.; Blair, K.A.; Peterson, D.E.; Kazmierczak, J.J.; Addiss, D.G.; Fox, K.R.; Rose, J.B.; et al. A massive outbreak in Milwaukee of *Cryptosporidium* infection transmitted through the public water supply.

N. Engl. J. Med. **1994**, *331*, 161–167.

30 Baldursson, S.; Karanis, P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010. *Water Res.* **2011**, *45*, 6603–6614.

31 Efstratiou, A.; Ongerth, J.E.; Karanis, P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks - An update 2011–2016. *Water Res.* **2017**, *114*, 14–22.

32 CDC (2017). Crypto outbreaks linked to swimming have doubled since 2014. https://www.cdc.gov/media/releases/2017/p0518-*Cryptosporidium*-outbreaks.html.

33 Izquierdo, J.; Antúnez, I.; Calderón, M.T.; Pérez Giraldo, C.; Muñoz Sanz, A. Diarrhea caused by *Cryptosporidium* and colonic neoplasia]. *Rev. Clínica Española* **1988**, *182*, 393–394.

34 de Souza, L. do R.; Rodrigues, M.A.M.; Morceli, J.; Kemp, R.; Mendes, R.P. Cryptosporidiosis of the biliary tract mimicking pancreatic cancer in an AIDS patient. *Rev. Soc. Bras. Med. Trop.* **2004**, *37*, 182–185.

35 Shebl, F.M.; Engels, E.A.; Goedert, J.J. Opportunistic intestinal infections and risk of colorectal cancer among people with AIDS. *AIDS Res. Hum. Retroviruses* **2012**, *28*, 994–999.

36 Hayward, A.R.; Levy, J.; Facchetti, F.; Notarangelo, L.; Ochs, H.D.; Etzioni, A.; Bonnefoy, J.Y.; Cosyns, M.; Weinberg, A. Cholangiopathy and tumors of the pancreas, liver, and biliary tree in boys with X-linked immunodeficiency with hyper-IgM. *J. Immunol.* **1997**, *158*, 977–983.

37 Stephens, J.; Cosyns, M.; Jones, M.; Hayward, A.R. Liver and bile duct pathology following *Cryptosporidium parvum* infection of immunodeficient mice. *Hepatology* **1999**, *30*, 27–35.

38 Leven, E.A.; Maffucci, P.; Ochs, H.D.; Scholl, P.R.; Buckley, R.H.; Fuleihan, R.L.; Geha, R.S.; Cunningham, C.K.; Bonilla, F.A.; Conley, M.E.; et al. Hyper IgM syndrome: a report from the USIDNET registry. *J. Clin. Immunol.* **2016**, *36*, 490–501.

39 Rahman, M.; Chapel, H.; Chapman, R.W.; Collier, J.D. Cholangiocarcinoma complicating secondary sclerosing cholangitis from cryptosporidiosis in an adult patient with CD40 ligand deficiency: case report and review of the literature. *Int. Arch. Allergy*

Immunol. **2012**, *159*, 204–208.

40 Osman, M.; Benamrouz, S.; Guyot, K.; Baydoun, M.; Frealle, E.; Chabe, M.; Gantois, N.; Delaire, B.; Goffard, A.; Aoun, A.; et al. High association of *Cryptosporidium* spp. infection with colon adenocarcinoma in Lebanese patients. *PLoS One* **2017**, *12*, e0189422.

41 Sulzyc-Bielicka, V.; Kuźna-Grygiel, W.; Kołodziejczyk, L.; Bielicki, D.; Kładny, J.; Stepień-Korzonek, M.; Telatyńska-Smieszek, B. Cryptosporidiosis in patients with colorectal cancer. *J. Parasitol.* **2007**, *93*, 722–724.

42 Sulżyc-Bielicka, V.; Kołodziejczyk, L.; Jaczewska, S., Bielicki, D.; Kładny, J.; Safranow K. Prevalence of *Cryptosporidium* sp. in patients with colorectal cancer. *Pol. Przegl. Chir.* **2012**, *84*, 348–351.

43 Sulżyc-Bielicka, V.; Kołodziejczyk, L.; Jaczewska, S.; Bielicki, D.; Safranow, K.; Bielicki, P.; Kładny, J.; Rogowski, W. Colorectal cancer and *Cryptosporidium* spp. infection. *PLoS One* **2018**, *13*, e0195834.

44 Kopacz, Ż.; Kváč, M.; Karpiński, P.; Hendrich, A.B.; Sąsiadek, M.M.; Leszczyński, P.; Sak, B.; McEvoy, J.; Kicia, M. The First Evidence of *Cryptosporidium meleagridis* infection in a colon adenocarcinoma from an immunocompetent patient. *Front. Cell Infect. Microbiol.* **2019**, *9*, 35.

45 Zhang, N.; Yu, X.; Zhang, H.; Cui, L.; Li, X.; Zhang, X.; Gong, P.; Li, J.; Li, Z.; Wang, X.; et al. Prevalence and genotyping of *Cryptosporidium parvum* in gastrointestinal cancer patients. *J. Cancer* **2020**, *11*, 3334–3339.

46 Patel, P.; Hanson, D.L.; Sullivan, P.S.; Novak, R.M.; Moorman, A.C.; Tong, T.C.; Holmberg; S.D.; Brooks, J.T.; Adult and adolescent spectrum of disease project and HIV outpatient study investigators. Incidence of types of cancer among HIV-infected persons compared with the general population in the United States, 1992-2003. *Ann. Intern. Med.* **2008**, *148*, 728–736.

47 Hunter, P.R.; Nichols, G. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. *Clin. Microbiol. Rev.* **2002**, *15*, 145–154.

48 Kalantari, N.; Gorgani-Firouzjaee, T.; Ghaffari, S.; Bayani, M.; Ghaffari, T.; Chehrazi M. Association between *Cryptosporidium* infection and cancer: A systematic review and

meta-analysis. *Parasitol. Int.* **2020**, *74*, 101979.

49 Uhl, E.W.; Jacobson, E.; Bartick, T.E.; Micinilio, J.; Schimdt, R. Aural-pharyngeal polyps associated with *Cryptosporidium* infection in three iguanas (Iguana iguana). *Vet. Pathol.* **2001**, *38*, 239–242.

50 Fitzgerald, S.D.; Moisan, P.G.; Bennett, R. Aural polyp associated with cryptosporidiosis in an iguana (*Iguana Iguana*). *J. Vet. Diagnostic Investig.* **1998**, *10*, 179–180.

51 Nakagun, S.; Horiuchi, N.; Sugimoto, M.; Tomikawa, S.; Watanabe, K.; Kobayashi, Y. Proventriculitis associated with *Cryptosporidium baileyi* in a snowy owl (*Bubo scandiacus*) and its epidemiological investigation. *J. Parasitol.* **2017**, *103*, 451–457.

52 Terrell, S.P.; Uhl, E.W.; Funk, R.S. Proliferative enteritis in leopard geckos (*Eublepharis macularius*) associated with *Cryptosporidium* sp. infection. *J. Zoo Wildl. Med.* **2003**, *34*, 69–75.

53 Certad, G.; Ngouanesavanh, T.; Guyot, K.; Gantois, N.; Chassat, T.; Mouray, A.; Fleurisse, L.; Pinon, A.; Cailliez, J.C.; Dei-Cas, E.; et al. *Cryptosporidium parvum*, a potential cause of colic adenocarcinoma. *Infect. Agent Cancer* **2007**, *2*, 22.

54 Benamrouz, S.; Guyot, K.; Gazzola, S.; Mouray, A.; Chassat, T.; Delaire, B.; Chabé, M.; Gosset, P.; Viscogliosi, E.; Dei-Cas, E.; et al. *Cryptosporidium parvum* infection in SCID mice infected with only one oocyst: qPCR assessment of parasite replication in tissues and development of digestive cancer. *PLoS One* **2012**, *7*, e51232.

55 Certad, G.; Creusy, C.; Guyot, K.; Mouray, A.; Chassat, T.; Delaire, B.; Pinon, A.; Sitja-Bobadilla, A.; Alvarez-Pellitero, P.; Praet, M.; et al. Fulminant cryptosporidiosis associated with digestive adenocarcinoma in SCID mice infected with *Cryptosporidium parvum* TUM1 strain. *Int. J. Parasitol.* **2010**, *40*, 1469–1475.

56 Certad, G.; Benamrouz, S.; Guyot, K.; Mouray, A.; Chassat, T.; Flament, N.; Delhaes, L.; Coiteux, V.; Delaire, B.; Praet, M.; et al. Fulminant cryptosporidiosis after near-drowning: a human *Cryptosporidium parvum* strain implicated in invasive gastrointestinal adenocarcinoma and cholangiocarcinoma in an experimental model. *Appl. Environ. Microbiol.* **2012**, *78*, 1746–1751.

57 Audebert, C.; Bonardi, F.; Caboche, S.; Guyot, K.; Touzet, H.; Merlin, S.; Gantois, N.;

Creusy, C.; Meloni, D.; Mouray, A.; et al. Genetic basis for virulence differences of various *Cryptosporidium parvum* carcinogenic isolates. *Sci. Rep.* **2020**, *10*, 7316.

58 Abdou, A.G.; Harba, N.M.; Afifi, A.F.; Elnaidany, N.F. Assessment of *Cryptosporidium parvum* infection in immunocompetent and immunocompromised mice and its role in triggering intestinal dysplasia. *Int. J. Infect. Dis.* **2013**, *17*, e593–600.

59 Mostafa, N.E.; Abdel Hamed, E.F.; Fawzy, E.M.; Zalat, R.S.; Rashed, H.E.; Mohamed S.Y. The new trend in the treatment of experimental cryptosporidiosis and the resulting intestinal dysplasia. *Color. Cancer* **2018**, *7*, CRC06.

60 Certad, G.; Creusy, C.; Ngouanesavanh, T.; Guyot, K.; Gantois, N.; Mouray, A.; Chassat, T.; Flament, N.; Fleurisse, L.; Pinon, A.; et al. Development of *Cryptosporidium parvum*-induced gastrointestinal neoplasia in severe combined immunodeficiency (SCID) mice: severity of lesions is correlated with infection intensity. *Am. J. Trop. Med. Hyg.* **2010**, *82*, 257–265.

61 Baydoun, M.; Vanneste, S.B.; Creusy, C.; Guyot, K.; Gantois, N.; Chabe, M.; Delaire, B.; Mouray, A.; Baydoun, A.; Forzy, G.; et al. Three-dimensional (3D) culture of adult murine colon as an *in vitro* model of cryptosporidiosis: Proof of concept. *Sci. Rep.* **2017**; *7*, 17288.

62 O'Hara, S.P.; Chen, X.M. The cell biology of *Cryptosporidium* infection. *Microbes Infect.* **2011**, *13*, 721–730.

63 Certad, G.; Viscogliosi, E.; Chabé, M.; Cacciò, S.M. Pathogenic Mechanisms of *Cryptosporidium* and *Giardia*. *Trends Parasitol.* **2017**, *33*, 561–576.

64 Schneider, S.; Weydig, C.; Wessler, S. Targeting focal adhesions: *Helicobacter pylori*-host communication in cell migration. *Cell Commun. Signal.* **2008**, *6*, 2.

65 Shostak, K.; Chariot, A. EGFR and NF-κB: partners in cancer. *Trends Mol. Med.* **2015**, *21*, 385–393.

66 Ding, L.; Gu, H.; Lan, Z.. Lei, Q.; Wang, W.; Ruan, J.; Yu, M.; Lin, J.; Cui, Q. Downregulation of cyclooxygenase-1 stimulates mitochondrial apoptosis through the NF-κB signaling pathway in colorectal cancer cells. *Oncol. Rep.* **2018**, *41*, 559–569.

67 Liu, T.L.; Fan, X.C.; Li, Y.H.; Yuan, Y.J.; Yin, Y.L.; Wang, X.T.; Zhang, L.X.; Zhao, G.H. Expression profiles of mRNA and lncRNA in HCT-8 cells infected with

Cryptosporidium parvum IId subtype. *Front. Microbiol.* **2018**, *9*, 1409.

68 Tiwari, A.; Saraf, S.; Verma, A.; Panda, K.; Jain, S.K. Novel targeting approaches and signaling pathways of colorectal cancer: An insight. *World J. Gastroenterol.* **2018**, *24*, 4428–4435.

69 Benamrouz, S.; Conseil, V.; Chabé, M.; Praet, M.; Audebert, C.; Blervaque, R.; Guyot, K.; Gazzola, S.; Mouray, A.; Chassat, T.; et al. *Cryptosporidium parvum*-induced ileo-caecal adenocarcinoma and Wnt signaling in a mouse model. *Dis. Model. Mech.* **2014**, *7*, 693–700.

70 Yamaoka, Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat. Rev. Gastroenterol. Hepatol.* **2010**, *7*, 629–641.

71 Xu, P.; Widmer, G.; Wang, Y.; Ozaki, L.S.; Alves, J.M.; Serrano, M.G.; Puiu, D.; Manque, P.; Akiyoshi, D.; Mackey, A.J.; et al. The genome of *Cryptosporidium hominis*. *Nature* **2004**, *431*, 1107–1112.

72 Puiu, D.; Enomoto, S.; Buck, G.A.; Abrahamsen, M.S.; Kissinger, J.C. CryptoDB: the *Cryptosporidium* genome resource. *Nucleic Acids Res.* **2004**, *32*, D329-331.

73 Hadfield, S.J.; Pachebat, J.A.; Swain, M.T.; Robinson, G.; Cameron, S.J.; Alexander, J.; Hegarty, M.J.; Elwin, K.; Chalmers, R.M. Generation of whole genome sequences of new *Cryptosporidium hominis* and *Cryptosporidium parvum* isolates directly from stool samples. *BMC Genomics* **2015**, *16*, 650.

2. Objectives and Strategies

Objectives

It is well known that interactions involving the pathogen and its host are critical in microbial pathogenicity. For this reason, the general objective of the thesis focuses on the study of interactions between *Cryptosporidium* and its host by combining diverse approaches to understand the pathophysiological aspects leading to the parasite initiated cancer

The specific objectives of the thesis are:

- To study the role of epigenetics on the interaction between *Cryptosporidium* and its host.
- To investigate signaling pathways involved in *C. parvum* induced digestive cancer in a mouse model.

Strategies

To address the parasite-host interaction we investigated firstly the parasite and its life cycle, followed by the study of the consequence of the infection in the host, and in particular the modulation of signaling pathways in association with cancer onset

1. To address the first aim, the epigenetic regulation called histone lysine methylation was investigated in both *Cryptosporidium* and the host cell. In particular, the enzymes called HKMTs catalyzing this mechanism were identified and characterized based on structural and functional analysis of the parasite. This was followed by the study of the impact of the infection on the host epigenome.

2. To address the second aim, the study of the dynamic changes that occur in the host cell after *C. parvum* infection was of significant interest. In this way, the global changes in the gene expression profile of *C. parvum* infected ileo-cecal regions of mice developing tumors were assessed using a microarray approach. The use of a web-based bioinformatics application called Ingenuity pathway analysis (IPA) further allowed analysis of potential molecular networks dictating the parasite stimulated cancer progression. Moreover, this study performed molecular characterization of tumor microenvironment induced by *C. parvum*. Potential signaling pathways found at the intersection of infection and cancer onset were also investigated.

Results

3. Results

3.1. Characterization of *Cryptosporidium* and host interactions through histone lysine methylation events.

This study is the subject of an article submitted to the journal "Virulence" and is under revision.

Title: **Putative SET-domain methyltransferases in *Cryptosporidium parvum* and histone methylation during infection**

Manasi Sawant, Sadia Benamrouz-Vanneste, Dionigia Meloni, Nausicaa Gantois, Karine Guyot, Colette Creusy, Erika Duval, René Wintjens, Jonathan Weitzman, Magali Chabe, Eric Viscogliosi, Gabriela Certad

My contribution to the study :

- Conceptualization of experiments
- Performing the experiments
- Analysis of the data
- Writing the manuscript

Overview :

Cryptosporidium is an Apicomplexan parasite that represents a major public health problem in a variety of vertebrates including humans. It is known to cause self-limited diarrhea in immunocompetent hosts and life-threatening diseases in immunocompromised hosts. Different cohort studies have reported it to be one of the four main pathogens responsible for severe diarrhea and mortality in children under 5 years old. Despite its prevalence and impact on public health, neither treatment nor vaccine against *Cryptosporidium* are yet available.

One of the ways to tackle this parasite, known as a public health problem, is by targeting its pathogenicity. Virulence factors affecting the host at any time during the life cycle are key aspects of this pathogenicity. These virulence factors have been identified in different developmental stages of the parasite indicating that *Cryptosporidium* indeed has a very dynamic transcriptome. Considering that *Cryptosporidium* has a relative paucity of transcription factors, epigenetics probably has an important role in the transcription regulation

contributing to parasite differentiation. Thus, the research presented herein focused on understanding the dynamic nature of histone lysine methylations. We began our work by performing *in silico* analysis to identify potential HKMTs of *Cryptosporidium*. Primary sequence alignment and phylogenetic analysis assisted in the identification of putative *C. parvum* HKMTs and their substrate specificities. Further, by performing homology modeling we predicted three structurally active HKMTs namely CpSET1, CpSET2 ,and CpSET8, and their functional significance was justified by observing histone lysine methylations such as H3K4Me3, H3K36Me3 and H4K20Me3 during intracellular development of *C. parvum*. Unlike other Apicomplexan parasites, it is difficult to perform *in vitro* cultivation of *Cryptosporidium* making it extremely difficult to readily knock out or knock down genes to see their effect on the parasite pathogenicity. To determine the importance of HKMTs on the pathogenicity of *Cryptosporidium*, we cloned and produced the putative HKMT, CpSET8 into a bacterial system and via an *in vitro* activity assay proved the existence of active HKMT in the parasite. Alongside, we also highlight the inherent capability of the parasite to exploit the host epigenome for its survival and persistence of the infection. Moreover, paving the way to utilize commercially available anti-HKMT drugs to fasten the research on yet unidentified treatment against the parasite. Thus, this study is the first one to provide insights on epigenetics mechanisms occurring throughout the parasite's life cycle and during the interaction with its host.

Results

Article 1

Putative SET-domain methyltransferases in *Cryptosporidium parvum* and histone methylation during infection

Manasi Sawant[1], Sadia Benamrouz-Vanneste[1, 2], Dionigia Meloni[1], Nausicaa Gantois[1], Gaël Even[3,4], Karine Guyot[1], Colette Creusy[5], Erika Duval[5], René Wintjens[6], Jonathan Weitzman[7], Magali Chabe[1], Eric Viscogliosi[1], Gabriela Certad[1, 8]

[1]Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France

[2]Equipe Ecologie et Biodiversité, Unité de Recherche Smart and Sustainable Cities, Faculté de Gestion, Economie et Sciences, Institut Catholique de Lille, France

[3]Gènes Diffusion, F-59501 Douai, France; g.even@genesdiffusion.com

[4]PEGASE-Biosicences

(Plateforme d'Expertises Génomiques Appliquées aux Sciences Expérimentales), Institut Past eur de Lille, F-59000 Lille, France

[5] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France

[6] Unit of Microbiology, Bioorganic and Macromolecular Chemistry, Department of Research in Drug Development (RD3), Faculté de Pharmacie, Université Libre de Bruxelles, 1050 Brussels, Belgium

[7] Université de Paris, Epigenetics and Cell Fate, CNRS, Paris, France.

[8] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France

[*] Correspondence: gabriela.certad@pasteur-lille.fr

Results

Abstract:

Cryptosporidium parvum is a major cause of an intestinal pathology called cryptosporidiosis which affects humans and other vertebrates. Despite being declared as a public health problem by World Health Organization (WHO) in 2006, pathogenesis caused by this parasite remains poorly understood. More recently, *C. parvum* has been linked with oncogenesis. In particular, the mechanisms involved in the processes of gene expression regulation are completely unexplored in *Cryptosporidium*. In the current study, we took the opportunity to investigate a dynamic epigenetic modification called histone lysine methylation during the life cycle of the parasite. We successfully identified putative SET-domain containing proteins, lysine methyltransferases (KMTs), which catalyze the methylation of different lysine residues. Phylogenetic analysis classified them into distinct subfamilies namely CpSET1, CpSET2, CpSET8, CpKMTox, and CpAKMT. Structural analysis further characterized CpSET1, CpSET2 and CpSET8 to be histone lysine methyltransferases (HKMTs). Their functional significance was predicted by using site-specific methyl-lysine antibodies during the development of the parasite (CpSET1:H3K4; CpSET2:H3K36; CpSET8:H4K20). In particular, the SET domain of CpSET8 showcased methyltransferase activity confirming the existence of functional HKMTs in *Cryptosporidium*. Moreover, the consequence of *C. parvum* infection on the host lysine methylation events highlights inherent potential of the parasite to exploit the host epigenetic regulation to its advantage. Thus, this study is the first one to provide insights on epigenetics mechanisms occurring throughout the parasite's life cycle and during the interaction with its host . As *Cryptosporidium* is a protozoan that significantly affects the health of both humans and animals, a better understanding of its developmental processes within the definitive host may highlight novel infection control strategies.

Keywords

Cryptosporidium, life cycle, histone lysine methyltransferases, epigenetic mechanisms, histone methylation, *C. parvum*-induced colon cancer

Results

Introduction

Cryptosporidium belongs to the eukaryotic phylum Apicomplexa, relative of the parasites that cause malaria and toxoplasmosis [1]. *Cryptosporidium* infection, also termed as cryptosporidiosis, is responsible for self-limited diarrhea in healthy immunocompetent individuals and can cause life-threatening disease in immunocompromised individuals [2]. A series of recent epidemiological studies reported that the diarrheal disease caused by the parasite is a leading cause of early childhood morbidity and mortality in developing countries, together with rotavirus, enterotoxigenic *Escherichia coli* and *Shigella* [3], [4]. The environmentally resilient nature of the *Cryptosporidium* oocysts allows the parasite to withstand common water treatments such as chlorination [5], and to remain a major cause of waterborne outbreaks in industrialized countries [6], [7]. Despite its significant impact on public health, there are currently no vaccine or chemoprophylactic drugs to prevent *Cryptosporidium* infection and very few chemotherapeutic options [8].

The majority of human infections by this protozoan are caused by *Cryptosporidium hominis* and *Cryptosporidium parvum*. Thus, new opportunities to uncover drug targets are attributed to the availability and *in silico* analysis of genome sequences of *C. hominis* [9] and *C. parvum* [10]. These two genomes are relatively small (approximately 9 Mb), adenosine- and thymidine-rich (approximately 70%) and differ at the nucleotide level by only 3-5% [9], [10]. In parallel, *C. parvum* transcriptomes at the oocyst, excysted sporozoites and intracellular stages have been widely investigated to understand *Cryptosporidium* life cycle development [11], [12], even though the asynchronous nature of the parasite life-cycle renders the transcriptome interpretations challenging. Interestingly, with the availability of the technologies to genetically manipulate the parasite [13], it became possible to analyze the transcriptome at specific intracellular stages *in vitro* as well as *in vivo* [14], leading to the discovery of novel targets for therapeutic interventions.

Strikingly, the compact genome of *Cryptosporidium* compared to other apicomplexan homologs (e.g approximately 63 Mb for *T. gondii*) accompanied by the lack of large families of recognizable transcription factors typically encountered in eukaryotic organisms [15], suggest major differences in the mechanisms of apicomplexan gene regulation. These mechanisms may depend on epigenetic events in order to control gene expression and cellular differentiation [16].

In eukaryotes, epigenetic changes include DNA methylation [17] and histone modifications such as lysine methylation [18] and acetylation [19]. Apicomplexan parasites such as

Results

Toxoplasma and *Cryptosporidium* are known to encode putative DNA methylation enzymes, but they lack detectable DNA cytosine methylation events [20]. On the other hand, novel drugs targeting the enzyme histone deacetylase (HDAC) which results in hyperacetylation of their genomes causing arrest of parasite differentiation has been reported in *Toxoplasma* [21], *Plasmodium* [22] and *Cryptosporidium* [23]. Along with acetylation, histone lysine methylation, a rather more sophisticated and dynamic post-translational modification previously identified to be restricted to metazoans has been extensively studied in *Toxoplasma* and *Plasmodium* [16]. Lysine methyltransferases (KMTs) known to methylate Histone 4 lysine 20 (H4K20), were identified to regulate cell-cycle progression in these two apicomplexan parasites [24]. In *Plasmodium*, lysine methylation markers such as Histone 3 lysine 4 (H3K4) and Histone 3 lysine 9 (H3K9) were reported in regulatory mechanisms of switching between variant surface antigens, enabling the parasite to evade the host immune response [25], [26]. Novel, parasite-specific methylation events might also be promising drug targets; for example, methylation of the parasite H3K18 residue was recently described in *Theileria* parasites [27]. Histone methylation is a reversible event that can be removed by histone demethylases. Jumonji-C-terminal (JmjC) domain-containing putative histone demethylases were identified in *T. gondii, P. falciparum, Babesia bovis,* and *Theileria annulata* [28]. Databases have also identified two lysine-specific demethylases (LSD)-like proteins in *T. gondii* genome [29]. However, these epigenetic modifications have not been previously investigated in *Cryptosporidium* parasites.

On the other hand, pathogens can induce alterations of their hosts employing several strategies to target cellular processes during their complex interactions with host cells [30]. In this way, they can evade the barriers imposed by checkpoint responses, and can manipulate various pathways activated for cell protection in order to increase their survival and transmission. Epigenetic mechanisms could play a fundamental role in the dynamic of host–parasite interactions [30]. As an example, lysine methylation is emerging as a versatile and dynamic post-translational modification that contributes critically to cellular differentiation programs being a pivotal dynamic event in host-pathogen interactions [31]. For example, *Theileria* parasites cause a lymphoproliferative disease in cows involving induction of host methyltransferases [31]. Strikingly, epidemiological and experimental studies suggest a potential link between *Cryptosporidium* infection and digestive cancer [32]. Of all apicomplexan parasites, *Cryptosporidium* and *Theileria* can transform their mammalian hosts. However, little is known about the significance of epigenetic variations in *Cryptosporidium*

development and in the parasite's interactions with its host.

In the current study, we aim to characterize the KMTs of *Cryptosporidium* in order to identify lysine methylation events which might be involved in regulating gene expression during the life cycle of the parasite and to evaluate host epigenetic events potentially involved in pathogenicity and parasite-induced transformation. Our study highlights the potential of lysine methylation modifications to be considered as targets in the development of therapeutic strategies against *Cryptosporidium* infection.

Materials and Methods

In silico analysis

The protein sequences of the SET domains of several representative KMTs including *Saccharomyces cerevisiae* SET1 (GenBank Accession number EDN62358) and SET2 (NP012367), *Homo sapiens* EZH2 (NP004447), SUV39H1 (BAD96791), SET8 (NP065115), SMYD3 (NP001161212), *Toxoplasma gondii* KMTox (XP002371399) and AKMT (XP 002370918) were retrieved from databases and used as queries to search *C. parvum* homologs by performing BLASTp analysis on the database CryptoDB (http://Cryptodb.org). In parallel, JumonjiC (JmJC)-domain was also used for the search of lysine demethylases (KDMs) in the *C. parvum* genome. The presence of the conserved SET domain within the putative KMTs of *C. parvum* was confirmed by analyzing the identified sequences using the InterPro program (http://www.ebi.ac.uk) which integrates the signatures provided from 13 different databases (CATH, CDD, HAMAP, MobiDB Lite, PANTHER, Pfam, PIRSF, PRINTS, PROSITE, SFLD, SMART, SUPERFAMILY and TIGRFAMs). Furthermore, multiple sequence alignment was performed to compare the putative SET domain sequences of *C. parvum* with those of a panel of 31 representative KMTs using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) software under manual supervision. Simultaneously, a phylogenetic analysis was performed from the same set of SET domain sequences. All positions containing gaps and regions of ambiguous alignment were removed, yielding 116 sites for phylogenetic inference. Full-length alignment and boundaries can be available upon request to the corresponding author. Briefly, phylogenetic trees were constructed using the Neighbour-joining (NJ) and Maximum Likelihood (ML) methods implemented in Mega X [33] using the Jones-Taylor-Thornton (JTT) substitution model. The relative stability of topological elements was assessed using 1000 bootstrap replicates for both NJ and ML.

Results

Homology modelling

Three-dimensional (3D) models of SET domains identified in the *C. parvum* genome (CpSETs) were built with the automated comparative modeling program Swiss Model Interactive Workspace (https://swissmodel.expasy.org/interactive) using as homologous protein templates, highly resolved X-ray crystal structures of human SET1 (MLL1) (Protein Data Bank (PDB) code: 5F6L.1 ; X-ray resolution 1.90 Å, SET2 (SETD2) (6J9J ; 1.78 Å), and SET8 (SETD8) (5TEG ; 1.30 Å). For each CpSET model developed, the quality of the structure was evaluated by the MolProbity web server [34]. The MolProbity score is a combination of the clash score, rotamer and geometric parameters, and the Ramachandran evaluations into a single score [35]. Lower MolProbity scores are better, meaning good quality structures. The MolProbity server reports also a percentile relative to the score distribution for crystal structures near the resolution of the submitted structure. In case of a modelled structure, the distribution is established covering all resolutions (range of 0Å-99Å). Distance matrix alignment (Dali) server [36] was used to perform pairwise structural alignment between the template and the newly generated CpSET models. The secondary structures were assigned using DSSP algorithm and the ChimeraX software was used to visualize the superimposition of templates and CpSET models [37].

Cryptosporidium oocysts

Oocysts of *C. parvum* strain Iowa (purchased from Waterborne™, New Orleans, LA, USA) were stored in phosphate-buffered saline (PBS) with penicillin, streptomycin, gentamycin, amphotericin B and 0.001% Tween 20 at 4°C until use. Absence of bacteria and fungi was assured by testing the oocyst suspensions on both Plate Count Agar and Sabouraud plates at 37°C for 1 week. Oocysts viability was determined as previously described [38].

In vitro culture

Human ileocecal adenocarcinoma cells (HCT-8; ATCC CCL-244) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 2 mM L-glutamine, 10% Fetal Bovine Serum (FBS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). Cells were cultured at 37°C in humidified incubator supplemented with 5% CO_2. Oocysts excystation was triggered as described previously [39]. After infecting the cells with the parasite, the culture was maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 15 mM HEPES buffer, 23 mM sodium bicarbonate, 5 mM glucose, 0.5 µM folic acid, 7 µM 4-aminobenzoic acid, 0.1 µM calcium pantothenate, 50 nM ascorbic acid, 1% (vol/vol) heat-

inactivated fetal calf serum, 210 µM gentamycin, 170 µM streptomycin and penicillin (105 U/mL). For negative controls that received no parasites, only maintenance medium was applied onto the monolayers.

Animal experiment

A total of 10 seven-week-old CB17-SCID mice were obtained from a colony bred at the Pasteur Institute of Lille (France). Mice were administered with 4 mg/L of dexamethasone (Merck, Lyon, France) through drinking water. Infective doses of *C. parvum* (10^5 oocysts / mouse) were prepared as described previously [40] and inoculated by oral-gastric gavage**.** In order to quantify parasite shedding, mice faeces were collected and treated as described previously [41]. At 60 days post-infection (PI) or when clinical signs of imminent death appeared, mice were euthanized by carbon dioxide inhalation. Experiments were conducted in the animal facility at the Institute Pasteur of Lille (research accreditation number, D 59 350 009). Animal protocols were approved by the French regional ethical committee with the number APAFIS#9621

Histopathology

Ileo-caecal regions were removed from each mouse, fixed in 4 % neutral formalin and embedded in paraffin. Sections of 4 µm thick were stained by hematoxylin-eosin-saffron (Leica Autostainer-XL, Rueil-Malmaison, France). Histological sections were analyzed using a Leica DMRB microscope equipped with a Leica digital camera connected to an Imaging Research MCID analysis system (MCID software, Cambridge, United Kingdom). Neoplastic lesions at different sites were scored as previously described [40].

Immunofluorescence assay

Sporozoites were fixed in 4% paraformaldehyde (PAF) for 10 min. After a wash with 1X PBS, the sporozoites were incubated in permeabilization solution (0.2 % Triton X-100 in 1X PBS) for 5 min then treated 10 min with blocking solution (0.3 M glycine, 1 % BSA, 0.1 % Tween 20 in 1X PBS). Finally, the sporozoites were incubated in primary antibody solutions for respective histone lysine methylations (Supplementary Table 1) overnight at 4 °C in a humidified chamber. The primary antibody solution was washed away with 1X PBS and the sporozoites were incubated with secondary antibody solution (Supplementary Table 1) for 1 h at room temperature. Following another wash with PBS, the sporozoites were incubated with the antibody anti-*Cryptosporidium (*Sporoglo, Waterborne™, New Orleans, LA, USA) for 45 min. After a final incubation with DAPI (1 µg/ml) for 15 min, the slides were mounted using

Mowiol mounting medium (Mowiol ® 4-88, Sigma, USA). For the *in vitro* staining, HCT-8 cells grown on coverslips in 24 well-plates were infected with 30,000 excysted oocysts per well and fixed at different time points PI: 6 h and 24 h to detect asexual stages and 55 h to detect sexual stages. The staining procedure was similar to that described above for sporozoites. For the *in vivo* staining, ileo-caecal sections of 5 µm thickness were obtained from formalin- fixed and paraffin-embedded specimens and placed on glass slides. The progressive rehydration was followed by an antigen retrieval step using citrate buffer pH 6.5 in a microwave oven for 15 min. After 1 h incubation in blocking buffer (2.5% BSA in 0.1 % Tween-20 1X PBS), the primary antibodies, diluted in blocking buffer, were applied for 1 h at 37°C. After three washes of 5 min with 1X PBS supplemented with 0.1 % Tween-20, the slides were incubated in the secondary antibodies for 1 h at 37°C. After a final wash, the slides were counterstained with DAPI and mounted with Mowiol mounting medium. The images were acquired using Zeiss LSM880 confocal microscope and analyzed using the ZEN lite Digital Imaging software.

RNA extraction, cDNA synthesis and Real-Time quantitative PCR (RT-qPCR)

Total RNA was extracted from infected and non-infected HCT8 cells using NucleoSpin RNA Kit (Macherey-Nagel, Germany). An on-column DNase digestion with a RNase-free DNase was included in the process described by the fabricant to remove any genomic DNA contamination in RNA samples. RNA quality and quantity were determined using Agilent RNA6000 Nano kit by capillary electrophoresis (Agilent 2100 bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). cDNA was synthesized from 1 µg of total RNA using oligo-dT primer and Superscript III reverse transcriptase (RT) in a 20 µl reaction (Invitrogen) according to the manufacturer's instruction. Each amplification was performed in a volume of 20 µl containing 1 µl of cDNA, 200 nM of each primer and 1X Brilliant III Ultra-Fast SybrGreen qPCR Master Mix (Agilent Technologies). The RT-qPCR reactions were performed on a QIAGEN Rotor-Gene Q instrument (Corbett Research, Qiagen) and included an initial denaturation at 95°C for 3 min followed by a two-step cycling protocol consisting of 45 cycles of denaturation at 95°C during 10 s and annealing/extension at 60°C during 10 s. The PCR cycling program was followed by a standard melt step, stepwise increasing temperature each 5 s by 1˚C, ranging from 65˚C to 95˚C. Primers used for RT-qPCR of putative CpKMTs are listed in supplementary Table 2. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of *KMT g*enes with the constitutively expressed 18S rRNA gene as the internal reference and the ΔC_t value of the sporozoite stage as the calibrator.

Results

Purification of histones and Western blot

Parasite histones were enriched by performing a fractionation protocol. Briefly, HCT-8 cells infected and non-infected at different PI time points were incubated in ice cold fractionation buffer (25mM Tris-HCl pH 8.5, 50 mM NaCl, 0.1 % Triton-X100, 1 mM EDTA, 1x protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche, USA). After dislocating the cells, the lysate was centrifuged at 2,000 g for 10 min at 4ºC. Respective cellular fractions (pellet and supernatant) were subjected to histone purification using the EpiQuik™ Total Histones Extraction Kit (Epigentek, OP-0006-100, USA). The purified histone concentration was determined using micro BCA protein assay kit (Pierce, Thermofischer Scientific, USA). Approximately equal amounts of purified histones and parasite histones were separated by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) and transferred to nitrocellulose membranes (Millipore, USA). Chemiluminescent detection of bands was carried out by using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA).

Cloning and protein expression of CpSETs

To express the SET domains proteins, putative active domains of CpSET1 (aa residues 2100 – 2244) and CpSET8 (aa residues 402 – 556) were *de novo* synthesized and cloned into the bacterial expression vector pET15b at the NdeI and BamHI cloning sites which follows N-terminal 6x histidine tag sequence (Gencust, Boynes, France). The expression plasmid was amplified in *E. coli* BL21 (DE3) cells. After induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16ºC for 16 h, the cells were collected by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM Imidazole, 1x protease inhibitor) and then broken using the bead beating method (Mixer Mill MM 400, Retsch, Germany). After centrifugation, his-tagged SET domains were recovered in the supernatant as soluble protein fractions.

***In vitro* histone methyltransferase assay**

To detect histone methyltransferase activity, 1 µg of recombinant H4 (New England Biolabs) and 100 µM S-adenosyl methionine (SAM) (Sigma, USA) were mixed with recombinant CpSET domains (CpSET8) in methyltransferase buffer (50 mM Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 4 mM DTT) at 30ºC for 1 h. The reaction was stopped by adding 2x SDS sample buffer, and the reaction mixture was analyzed on 15% SDS-PAGE, followed by western blotting with

antibodies against tri-methylation of lysine 20 of histone 4 (H4K20me3) at a dilution of 1:1000 (Supplementary table 1).

Signal quantification

Fluorescence intensity signals between infected and non-infected host tissue were quantified using ImageJ software version 1.52a (NIH, USA). Signal intensity was measured from the individual infected vs non-infected epithelial cells from intestinal crypts. For the statistical analysis, a mixed model was used to test the relationship between fluorescence intensity markers and group conditions taking into account sample repetition. A mixed regression model was created considering fluorescence quantification as the main outcomes and sample identifier as random effect. The general significance level was set at a p-value below 0.05. All analyzes were performed using packages nlme from the R statistical computing program (Version 4.1.1, date of release 8 October 2021; R Development Core Team, http://www.R---project.org, accessed on 12 January 2022) The data is represented using GraphPad Prism 9.1 (San a Diego, California, USA)

Results

Results

In silico identification of putative KMTs of *C. parvum* and sequence comparison of their SET domains

To identify putative KMTs in the *C. parvum* genome, we ran queries to retrieve genes from *C. parvum* containing SET-domain consensus. We identified ten putative KMTs with a recognisable SET domain (Table 1).

Table 1 - List of putative KMTs identified in the *C. parvum* genome

Gene ID	Name	Chromosomal location	Protein size (amino acids)	Domains in addition to SET domain	Family representative	Predicted Substrate Specificity
cgd8_2730	CpSET1	CM000436: 718307..725208 (+ strand)	2244	PHD, Bromo, post-SET	SET1	H3K4Me1/2/3
cgd5_400	CpSET2	CM000433: 103870..106923 (+ strand)	1004	Zinc finger, PHD, AWS, post-SET	SET2	H3K36Me1/2/3
cgd4_370	CpSET8	CM000432: 90500..92170 (+ strand)	556	-	SET8	H4K20Me1/2/3
cgd4_2090	CpAKMT	CM000432: 502573..504324 (- strand)	583	TPR	AKMT	Unknown
cgd5_2340	CpKMTox	CM000433: 503754..505532 (+ strand)	524	-	KMTox	H2A/H4
cgd7_5090	CpSET UNK1	CM000435: 1177390..1179244 (- strand)	574	Zinc finger - MNYD	SMYD3 ?	H3K4 ?
cgd1_2170	CpSET UNK2	CM000429: 494029..495242 (- strand)	366	-	SMYD3 ?	H3K4 ?
cgd6_1470	CpSET UNK3	CM000434: 350153..352792 (- strand)	879	AWS	Ezl1p ?	H3K27 ?
cgd8_3840	CpSET UNK4	CM000434: 894260..896419 (+ strand)	719	-	?	?
cgd6_980	CpSET UNK5	CM000434: 248189..249592 (- strand)	467	-	?	?

*PHD – Plant homeodomain
*AWS – Associated with SET
*TPR – Tetrapeptide repeat
*UNK – Unknown

Results

The corresponding genes are distributed on 6 chromosomes and the size of the full-length proteins range from 467 to 2244 amino acids. *In silico* analysis also showed the presence of CpSET proteins orthologues in all *Cryptosporidium* species including *C. muris, C. andersoni, C. hominis, C. meleagridis* and *C. ubiquitum*. Among the known methylated lysines, only H3K79 is known to be methylated by DOT1, a non-SET domain histone methyltransferase in mammalians. Unlike SET domain proteins, DOT1 domain containing proteins were observed only in *C. muris* and *C. andersoni*. Analysis of the domain organisation of so-called CpKMTs identified additional domains, including the bromodomain, plant homeodomain (PHD), associated with SET (AWS) domain, tetratricopeptide repeat (TPR) domain and MYND (Myeloid translocation protein 8, Nervy, and DEAF-1) (Table 1) (Figure 1). In contrast, we did not identify any *Cryptosporidium* genes with JmJC-domains which are characteristic of lysine demethylases (KDMs).

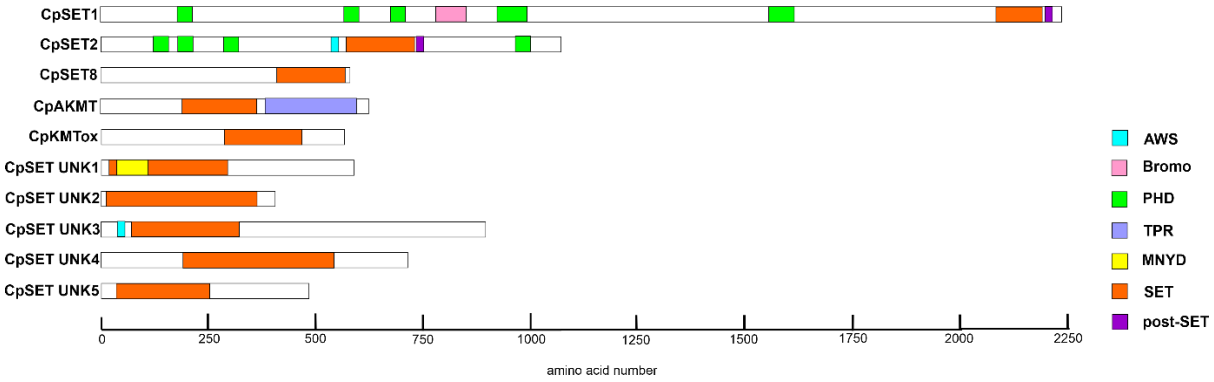


Figure 1. Schematic representation of putative lysine methyltransferases. The image illustrates SET and non-SET domain organizations in 10 putative KMTs of *C. parvum*. The additional domains include, associated with SET (AWS), bromodomain, plant homeodomain (PHD), tetratricopeptide repeat (TPR), Myeloid translocation protein 8, Nervy, and DEAF-1 (MYND). The schematic view of different SET domains varies due to the presence of variable region between motif II and motif III.

The mammalian SET-domain proteins are classified into seven families including SUV39, SET1, SET2, EZ, RIZ, SMYD and SUV4-20, as well as a few orphan members SET7/9 and SET8 (also called as Pr-SET7). We aligned sequences of CpSET domains with representatives of each of these families. Sequence analysis of SET domains of KMTs revealed the presence of four signature motifs; motif I (GxG), motif II (YxG), motif III (RFINHxCxPN) and motif IV (ELxFDY) (Figure 2). Our analysis showed that 8 out of 10 predicted CpSET proteins exhibited at different levels of similarity of the three catalytically essential motifs GxG, RFINHxCxPN and ELxFDY of the SET domain (Figure 2). This was not the case for the two

remaining CpSETs (cgd6_3840 and cgd6_980), which were excluded from further analysis. The motif II is very well conserved in cgd8_2730, cgd5_400, and cgd4_370 compared to other CpSETs. The sequence between motif II and motif III was observed to be the most variable region of the SET domain. The canonical preSET domain was not found preceding any of the CpSETs according to InterPro database. However, cysteine (Cys) rich cluster was observed flanking the N-terminal extremity of cgd5_400 and cgd6_1470 SET domains. This region has been identified as an AWS domain by the InterPro database. Regarding the C-terminal flanking region of the CpSET domain, it is composed of the post-SET domain which contains the $CXCX_{2\text{-}4}C$ motif, well conserved in cgd8_2730, cgd5_400, cgd1_2170, and cgd6_1470. This region has also been identified as post-domain in InterPro database. All the CpSETs exhibiting the post-SET domain also conserved the Cys residue in motif III. Indeed, the Cys residues from the post-SET domain and motif III together form a channel to accommodate the target of the lysine side-chain. However, cgd7_5090 showed sequence variation in the post-SET motif ($CXCX_2C$) similar to SMYD (SET-and MYND-domain containing) and Suv4-20 KMT families. With distinct residues conserved in motif III, cgd5_2340 is recognized to be part of separate family of KMT called KMTox. Finally, cgd4_2090 was identified to conserve the post-SET cysteine cluster ($CXCX_2CX_{11}CX_2C$) which has been previously described to be a feature of Apical lysine methyltransferases (AKMT), a cluster of KMTs only including apicomplexan homologs (Figure 2).

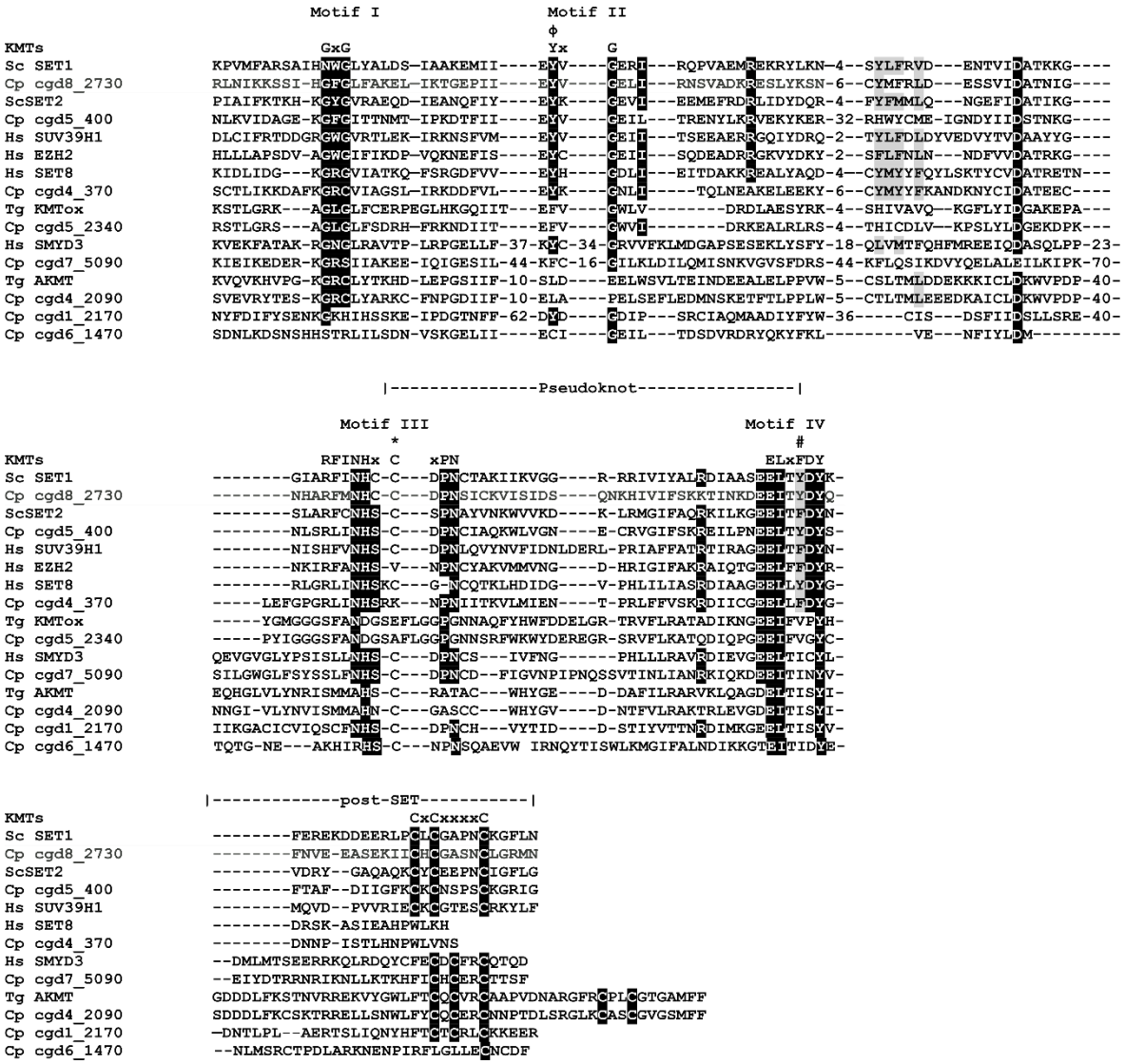


Figure 2. Alignment of SET and post-SET domain sequences of putative CpKMTs with representatives of KMT families. The SET domain is divided into four motifs (I-IV) and their consensus sequences are indicated above the alignment. Motif III and IV are involved in formation of pseudoknot structure to form the active site. The white text on black background indicates identical residues; black text on gray background indicates conserved residues. The residues representing catalytic site are indicated with phi (ϕ). The residues representing the F/Y switch are indicated with a hash symbol (#). The Cys residue from Motif III that is involved in Zn cluster formation with the post-SET domain is indicated with asterisks (*).

Results

To classify the parasite KMTs, we performed a phylogenetic analysis of the 8 *C. parvum* putative CpSETs, comparing them with representatives of different substrate-specific SET domains from yeast, *Drosophila*, humans, and Apicomplexa species (Figure 3). We are aware that phylogeny alone is not a reliable predictor of function, but the grouping of the CpSETs gives clues that might assist in subsequent functional analysis. cgd8_2730 clustered with representatives of the SET1 family of HKMTs such as *S. cerevisiae* SET1 and *H. sapiens* SET1 and was thus assigned it as CpSET1. This distribution was strongly supported by bootstrap resampling in NJ (86%) and ML (97%) methods. The clustering of CpSET1 together with previously identified apicomplexan homologs from *T. gondii*, *P. falciparum* and *T. annulata* was also moderately supported by bootstrap values (51% and 65% of the replicates under NJ and ML, respectively). The substrate specificity of this family is at the 4th lysine residue of the histone 3 protein (H3K4). Thus, we predicted H3K4 might be the substrate of CpSET1 (Figure 3) (Table 1).

The cgd5_400 gene sequence fell within the paraphyletic SET2 family of KMTs including *S. cerevisiae* and *H. sapiens* as well as apicomplexan enzymes from *T. gondii*, *T. annulata* and *P. falciparum*. Our phylogenetic tree suggested that cgd5_400 belongs to the SET2 family of KMTs which have been reported to methylate H3K36. We assigned cgd5_400 as CpSET2 (Figure 3) (Table 1).

The cgd4_370 gene was observed to be highly homologous to human SET8 and therefore named CpSET8. This homology was strongly supported by bootstrap values of 87% and 94% according to NJ and ML methods, respectively, in our phylogenetic analysis, suggesting that CpSET8 might methylate H4K20 as described for human SET8. Another putative KMT of *C. parvum* cgd5_2340 grouped together with KMTox from the Apicomplexa *T. gondii* and *Besnoitia besnoiti* with strong bootstrap values of 99% (NJ method) and 98% (ML method) and was thus assigned as CpKMTox. KMTox was defined as a new family of nuclear KMTs specifically found in Apicomplexa and thus including *Cryptosporidium*. Similar to KMTox, AKMTs also form a distinct clade from other known KMTs only found in apicomplexan species. cgd4_2090 represents *Cryptosporidium* AKMT (CpAKMT) as it clustered with high bootstrap support (bootstrap values of 99% and 99% according to NJ and ML methods, respectively) with other apicomplexan homologues.

The phylogenetic emergence of the three remaining CpSETs, named CpSET Unk1 (cgd7_5090), CpSET Unk2 (cgd1_2170) and CpSET Unk3 (cgd6_1470), remained uncertain according to our present study (Table 1). Indeed, CpSET Unk3 branched with unsupported bootstrap support at the base of a large group including KMT of the protozoan ciliate

Tetrahymena and the KMTox of Apicomplexa. Since the KMT of *Tetrahymena thermophila* is known to methylate H3K27, CpSET Unk3 could be involved in the same methylation event even if no sequence has been yet reported to be associated with H3K27 substrate specificity in Apicomplexa (Figure 3).

The two others CpSET Unk1 and Unk2 are representative of a paraphyletic group that includes in particular the human KMT SMYD3 first described to methylate H3K4 (and subsequently H4K5 and H4K20). The weak grouping of CpSET Unk1 and CpSET Unk2 with SMYD3 implies a possible methylation of H3K4 by these two KMTs of *Cryptosporidium* (Figure 3).

To complete this overview, unlike other apicomplexan parasites, no *C. parvum* sequence was found to be associated with the SUV39 and EZ families mediating H3K9 and H3K27 methylation, respectively (Figure 3).

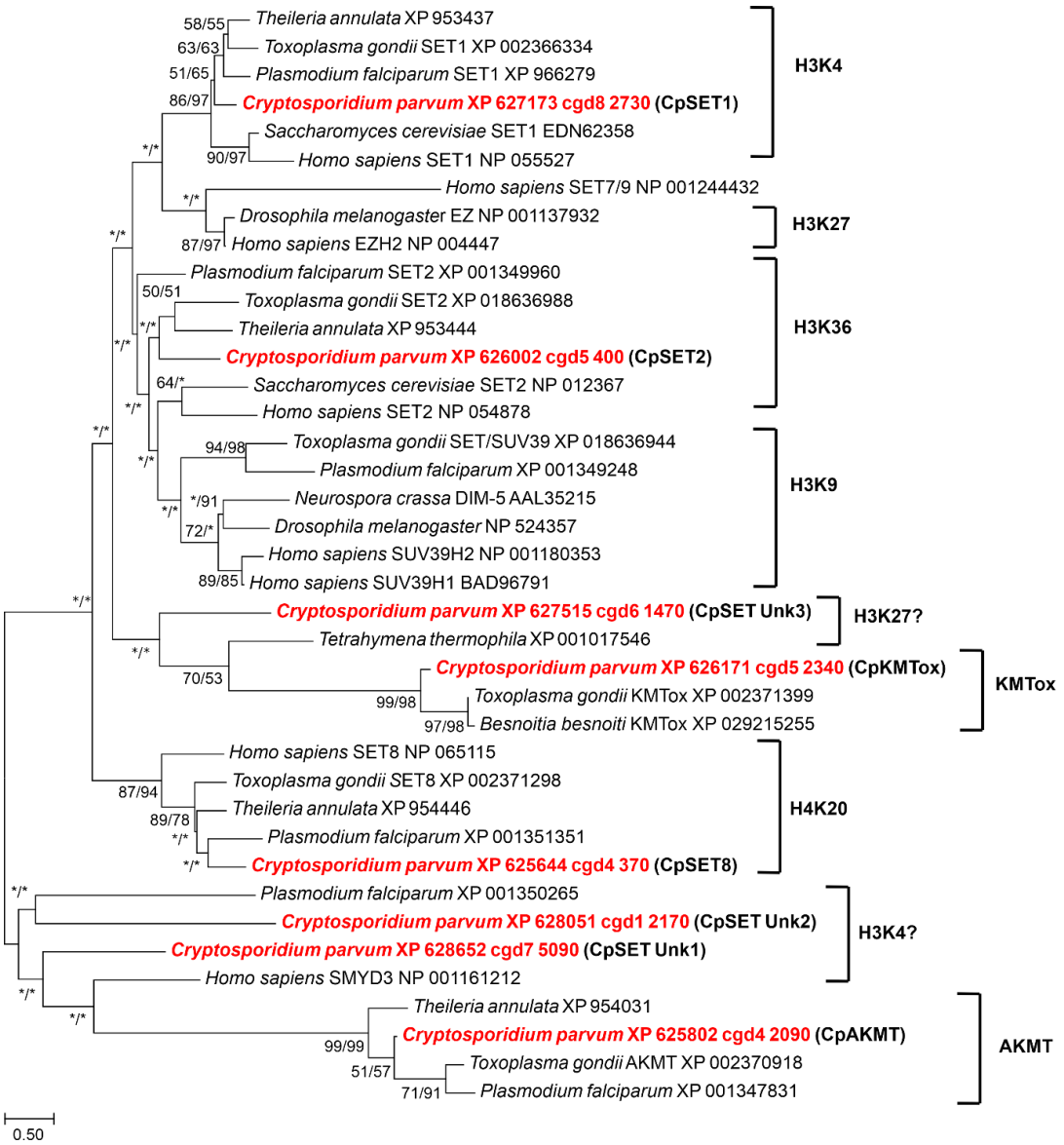


Figure 3. Phylogenetic analysis of SET domain proteins of *C. parvum*. All the putative *Cryptosporidium* sequences are highlighted in red. Numbers near the individual nodes indicate bootstrap values given by NJ (left of the slash) and Maximum Likelihood (right of the slash). Asterisks indicate nodes with bootstrap values below 50%. Branch lengths are proportional to sequence divergence and can be measured relative to the scale bar. The scale bar indicates the branch length corresponding to 0.50 substitutions per site. The predicted substrate specificities of KMTs are also indicated on the right of the figure. The substrate specificities of characterized KMTs clustering with the CpSET domain proteins are also indicated on the right of the figure. Except KMTox and AKMT are not "substrate specificities"

Results

Structural characteristics of CpSETs

Homology modelling of the structures of CpSET1, CpSET2 and CpSET8 using publicly available X-ray crystal structures of homologous enzymes showed that the overall architecture of the SET domains belonging to different subfamilies of KMTs were nearly identical. The MolProbity scores evaluating the quality of the models were good with values of 1.62 (92nd percentile), 1.73 (88th percentile), and 1.57 (93rd percentile) for CpSET1, CpSET2 and CpSET8, respectively (Table 2).

Table 2 - MolProbity statistics for 3D models of CpSETs

3D model	Clashscore[a]		Ramchandran favored	MolProbity Score[b]	
CpSET1	2.24	99th percentile	95.04%	1.62	92nd percentile
CpSET2	2.91	98th percentile	89.22%	1.73	88th percentile
CpSET8	4.61	96th percentile	94.52%	1.57	93rd percentile

[a] percentile score established with N =1784, considering all resolutions

[b] Percentile score established with N=27675, considering structures at all resolution ranges

Pairwise structure comparisons with the DALI program was used to check whether conserved residues line up between CpSETs and the templates. As described in Table 3, CpSET1 shares highest structural identity with the templates (52%) compared to CpSET2 (43%) and CpSET8 (44%). The visualization of these superimposed structures was performed using ChimeraX software (Figure 4).

Briefly, all the structures contained the specific β fold identified only in KMTs, not in any other previously characterized AdoMet-dependent methyltransferases. The fold has several series of curved β strands forming several small sheets that define the core of the SET domain. This β fold is followed by knot-like structure which is also observed in all the 3D models of CpSETs. The knot involves a C-terminal β strand threading through a hoop consisting of two β strands and their connecting region. This represents an archetypal feature of SET domain which consists of residues from the motif II, motif III and post-SET region that enclose the lysine residue and holds it in the appropriate chemical environment and position for methyl transfer by motif I (Figure 3, boxed in black dotted line). The essential residues have a similar arrangement in all the SET domains (Figure 4, enlarged images of active site).

Results

CpSET1 (Tyr 2129 and Tyr 2217) and CpSET2 (Tyr 574 and Tyr 659) conserved the key tyrosine residues (Figure 3A; 3B). These Tyr residues are expected to form an intricate network of hydrogen bonds which would place the methyl group in direct line with the N_{ε} of the lysine residue of the histone tail. Structural alignment revealed that in CpSET8 one of these tyrosine residues is replaced by phenylalanine residue (Tyr 445 and Phe 537) (Figure 4C). This represents the F/Y switch which determines whether the KMT can mono-, di- or tri- methylate the histone.

The C-terminal flanking region of different SET domain families is often divergent but CpSET1 and CpSET2 exhibited a classical post-SET domain. The prominent feature of this domain is a zinc-binding cage formed by three Cys residues from the C-terminal region whereas the fourth tetrahedral Cys ligand (CpSET1 Cys 2183 ; CpSET2 Cys 654) is provided by the loop linking motif II and motif III of SET domain. The narrow channel formed as a result of Cys interactions accommodates the target lysine and brings the Nε in close proximity of the donor at the opposite end of the channel (Figure 4A, B). In CpSET8, the C-flanking domain consists of a helix and the presence of the Trp 552 residue is responsible for interactions with the cofactor (Figure 4C).

Table 3. Pairwise structure comparison between generated models and templates

Structure / **Parameters**	**CpSET1 onto 5F6L**	**CpSET2 onto 6J9J**	**CpSET8 onto5TEG**
RMSD (Å)[a]	0.3	0.6	0.4
Number of superimposed residues	136	139	144
Total number of residues	143	169	148
Dali z-score[b]	24.4	22.2	25.8
Percentage identity	52 %	43 %	44 %

[a] RMSD is the root-mean-square deviation computed over the Cα atoms of superimposed residues.

[b] A DALI z-score greater than 3 is considered as reflecting similar structures. Higher z-scores indicate more similar structures.

[c] the percentage identity is calculated between structurally superimposed residues.

Results

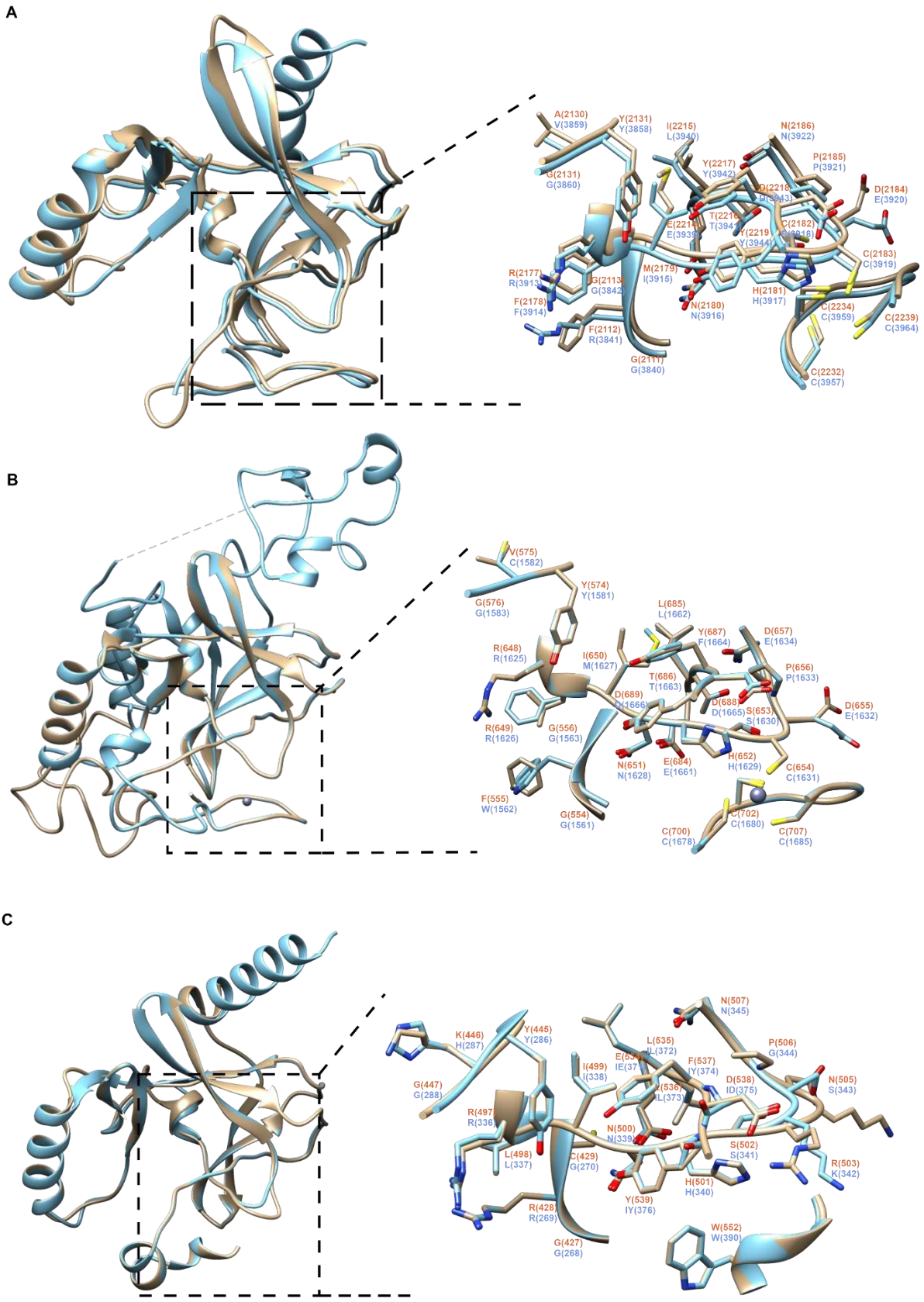

A
A(2130) V(3859)
Y(2131) Y(3858)
I(2215) L(3940)
N(2186) N(3922)
P(2185) P(3921)
Y(2217) Y(3942)
G(2131) G(3860)
D(2184) E(3920)
C(2183) C(3919)
M(2179) I(3915)
R(2177) R(3913)
F(2178) F(3914)
N(2180) N(3916)
H(2181) H(3917)
C(2234) C(3959)
C(2239) C(3964)
F(2112) R(3841)
G(2111) G(3840)
C(2232) C(3957)
B
V(575) C(1582)
Y(574) Y(1581)
G(576) G(1583)
L(685) L(1662)
Y(687) F(1664)
D(657) E(1634)
R(648) R(1625)
I(650) M(1627)
P(656) P(1633)
T(686) T(1663)
D(689) D(1666)
D(655) E(1632)
S(653) S(1630)
R(649) R(1626)
G(556) G(1563)
N(651) N(1628)
E(684) E(1661)
H(652) H(1629)
C(654) C(1631)
F(555) W(1562)
G(554) G(1561)
C(702) C(1680)
C(700) C(1678)
C(707) C(1685)
C
N(507) N(345)
K(446) H(287)
Y(445) Y(286)
L(535) L(372)
P(506) G(344)
F(537) IY(374)
G(447) G(288)
D(538) ID(375)
N(505) S(343)
R(497) R(336)
N(500) N(339)
L(498) L(337)
C(429) G(270)
S(502) S(341)
R(503) K(342)
H(501) H(340)
Y(539) IY(376)
R(428) R(269)
W(552) W(390)
G(427) G(268)

Figure 4. Structural modelling of SET domain regions of *C. parvum* KMTs**.** Superimposition of 3D homology models of SET domains of [sandy brown] CpSET1 (A), CpSET2 (B) and CpSET8 (C), coloured in sandy brown, with, in cyan, those of resolved crystal structures of SET domains of *Homo sapiens* , HsSET1, HsSET2 and HsSET8, respectively. The superimposition of the catalytic site has been highlighted in the black dotted box and depicted in an enlarged view with side chains showed in stick representation (nitrogen and oxygen atoms in blue and red, respectively) and labelled in orange and blue for *C. parvum* and *H. sapiens* structures, respectively.

Functional analysis of a putative *Cryptosporidium* SET-domain enzyme, CpSET8

To investigate whether the CpSETs identified in our *in silico* and phylogenetic analysis represent true active KMTs, we produced SET-domain regions of CpSET1 and CpSET8 in a bacterial expression system. Western blots analysis showed detectable amounts of CpSET8 in the soluble fraction after induction and lysis of bacteria (this was not the case for CpSET1) (Figure 5A). We performed a KMT catalytic activity assay, with recombinant Histone H4, on the induced soluble protein fraction of bacterial lysate containing CpSET8 and observed a relatively strong band around 15kDa (Figure 5C, Lane 2) compared to the controls without SAM (Figure 5C, Lane 1) or H4 (Figure 5C, Lane 3). The specificity of H4K20 methylation band was confirmed by the detection of H4 at the same molecular weight. Moreover, since anti-his tag antibody detects the 6X histidine tag present in the recombinant CpSET8 and, equal intensity of anti-His tag bands corresponds to equal amount of protein loaded in each well, this indicated that the high intensity band detected (red arrow) was the result of enzymatic activity of CpSET8 (Figure 5C).

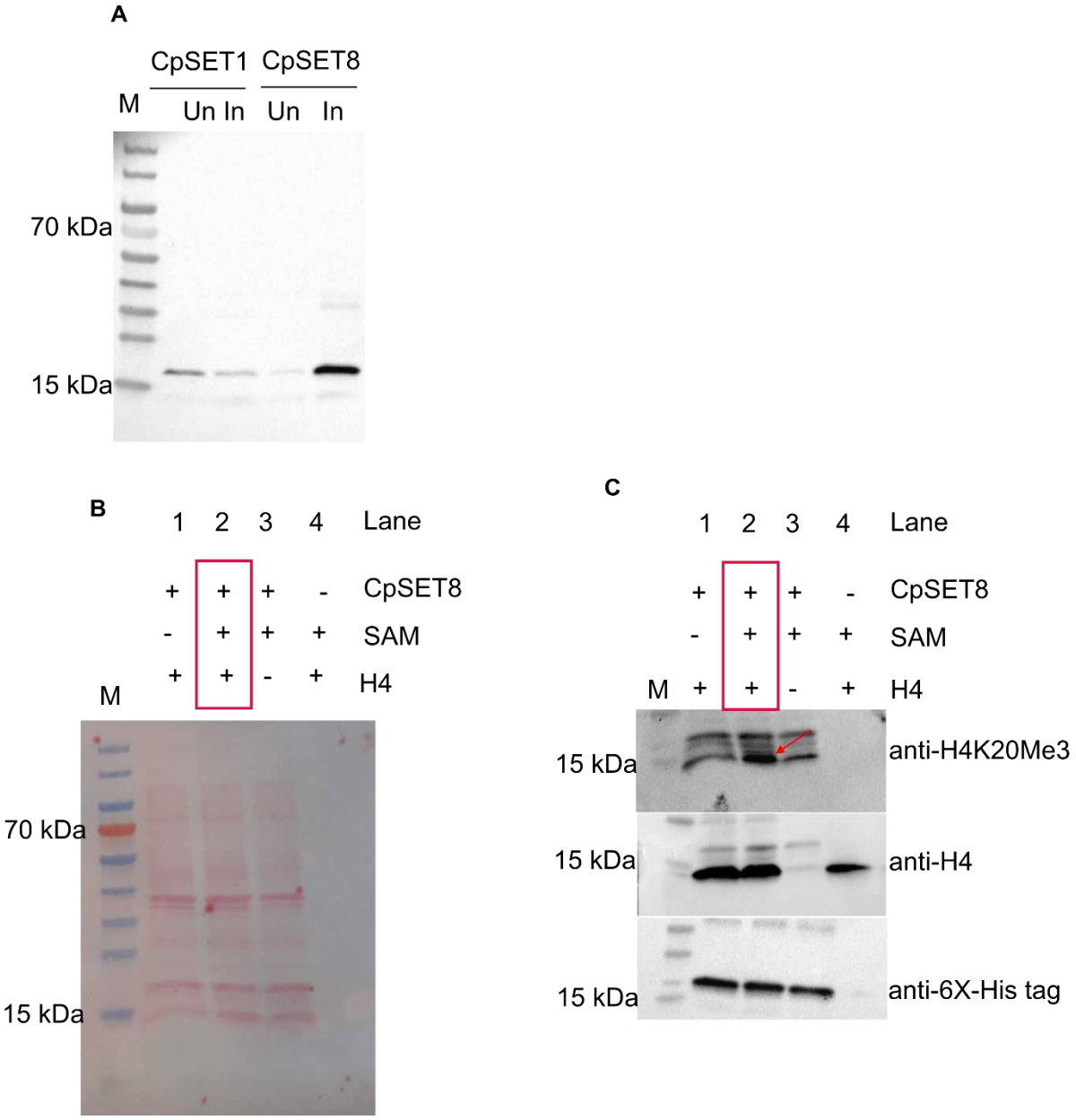


Figure 5. Analysis of recombinant CpSET8 enzymatic activity. (A). Western blot analysis of 6x-histidine tagged CpSET domains using a bacterial expression system. Anti-6x-His tag antibody was used to detect the CpSETs. M – Molecular marker, Un – Uninduced soluble protein fraction, In – Induced soluble fraction. (B). Ponceau S staining of the Western blot subjected to chemiluminescent detection of H4 methylation by recombinant 6x-histidine tagged CpSET8. (C). Chemiluminescent signals showing CpSET8 to tri-methylate H4, using anti-H4K20me3 antibody (red arrow). Recombinant human H4 was detected using anti-H4 antibody. CpSET8 was detected using anti-6x-His tag antibody. H4 – Histone 4. SAM – S-adenosyl methionine.

Results

Analysis of parasite KMTs and histone methylation during the *C. parvum* life cycle

To determine the expression pattern of CpSET KMTs during the *C. parvum* biological cycle *in vitro*, we performed RT-qPCR analysis of the eight *CpSET* genes. The expression of all eight *CpSET* genes was determined at 3 time points, i.e. 6 h, 24 h and 55 h PI (intracellular stages), relative to their expression at 2 h PI (extracellular sporozoite stage). *CpSET1* was the highest expressed KMT gene, particularly during initial stages of the infection; *CpSET1* showed a 14-fold increase at 6 h PI, corresponding to the time at which trophozoite development is predominant. *CpAKMT* expression was 10-fold increased during meront development at 24 h PI. *CpSET2*, *CpSET8,* and *CpKMTox* genes showed relatively high expression during trophozoite stage (6 h PI) followed by asexual (24 h PI) and sexual stages (55 h PI). At 55 h PI, wherein the sexual stages were predominant, all the identified putative *CpKMTs* were constitutively expressed. The gene expression of putative unknown *CpKMTs,* such as *CpSET Unk1*, *CpSET Unk2* and *CpSET Unk3,* during intracellular development was observed to be lower compared to that observed during the extracellular sporozoite stage at 2 h PI (Figure 6A).

The orthologues of the putative CpKMTs, CpSET1, CpSET2 and CpSET8 have been identified as histone lysine methyltransferases (HKMTs). In order to functionally characterize these HKMTs, we analyzed the histone lysine methylation events associated with their activity during the life cycle of *C. parvum in vitro* with relation to the different parasite developmental stages. Importantly, in *C. parvum* the sequences of the histone N-terminal tails, especially the Lysine residues, in the H3 and H4 proteins are extremely well conserved, compared to human sequences (supplementary Figure 1). This allowed us to utilize an array of commercial antibodies recognizing different modified lysine residues of H3. As previously described, CpSET1, CpSET2 and CpSET8 are orthologues of SET-domain proteins targeting H3K4, H3K36 and H4K20 methylation (Figure 3). Immunofluorescence analysis revealed that H3K4, H3K36 and H4K20 methylation is observed during all the intracellular stages of the parasite. We observed that anti-tri-methyl H3K4 (H3K4me3) antibodies recognized the *C. parvum* chromatin with a broader nuclear distribution through all the developmental stages. Immunofluorescence analysis with anti-tri-methyl H3K36 (H3K36me3) and anti-tri-methyl H4K20 (H4K20me3) antibodies staining showed a punctate pattern spread throughout the nucleus, most likely the pericentric heterochromatin during the intracellular stages compared to extracellular sporozoite stage (Figure 6B). We quantified the labelling of histone lysine methylation by western blotting analysis (Supplementary Figure 2A). We confirmed that

Results

H3K4 methylation levels remain consistent during meront development at 24 h PI and microgamont and macrogamont development at 55 h PI. On the other hand, H3K36 and H4K20 methylation levels fluctuated from asexual (24 h PI) to sexual stages 55 h PI (Supplementary Figure 2A). H3K36 and H4K20 methylation increased by 2-3 fold, when there was a predominance of sexual stages of the parasite in the culture (Supplementary Figure 2B).

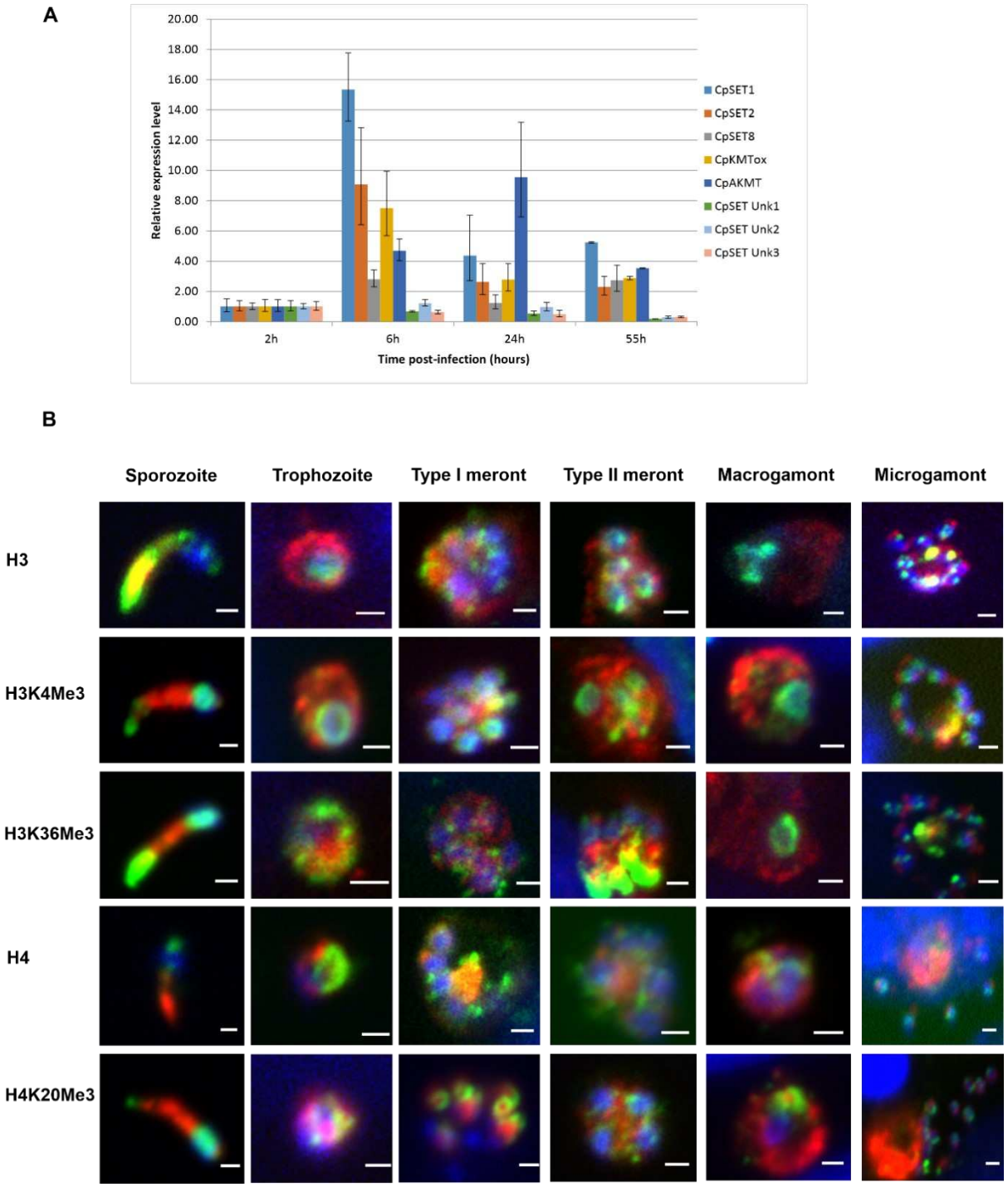


Figure 6. Characterization of KMT expression and histone methylation during *C. parvum* infection in *in vitro* culture. (A). RT qPCR analysis illustrating expression of *CpSET* genes during *C. parvum* development *in vitro*. The expression levels were analyzed in triplicates and normalized with 18S rRNA gene as the internal control. The ΔC_t values at the sporozoite stage were used as the calibrator. (B). Immunofluorescence analysis of histone lysine modifications in different stages of *C. parvum*. Co-staining with anti-histone antibodies (H3 and H4) and anti-histone methylation antibodies H3K4me3, H3K36me3 and H4K20me3 (green), anti-*Cryptosporidium* antibody (red) and DAPI (blue).

Results

C. parvum infection impacts the methylation of lysine residues in host histones

The well-documented model of *C. parvum*-induced colon cancer in SCID mice treated with dexamethasone offers an opportunity to study the impact of infection of host cell methylation *in vivo*. We detected *C. parvum* infection in this animal model and was confirmed by quantification of the oocyst shedding for the entire duration of the experiment. Upon histological examination of the ileo-caecal region of infected animals, the presence of well-differentiated adenocarcinomas invading the submucosae through the *muscularis mucosae* was confirmed after 60 days PI. We performed immunofluorescence analysis to examine the effect of *C. parvum* on an array of histone modifications. As previously described (Wang et al. 2017), the repressive H3K9me3 chromatin mark was upregulated in the epithelium of the ileo-caecal region of *C. parvum*-infected animals at 60 days PI (Supplementary Figure 3). In contrast, we observed downregulation of the activating methylation mark H3K4me3 (Supplementary Figure 3) in infected hosts. Our most striking finding was the marked deregulation of well-conserved methylation marks associated with transcriptional elongation (H3K36me3) and gene repression (H3K27me3) following infection. Immunofluorescence analysis revealed that H3K27me3 and H3K36me3 marks decreased significantly in the epithelium of the ileo-caecal region of *C. parvum* infected animals at 60 days PI (Figure 7A, B). In particular, these two methylations were significantly downregulated in the intestinal crypts where the presence of the parasite was detected (Figure 7A, red arrows). Interestingly, we obtained similar results during *C. parvum* infection of epithelial cells *in vitro.* At 55h PI, *C. parvum* can be observed in all the developmental stages, and the methylation marks were significantly downregulated in infected HCT8 cells (Figure 7C, D). Thus, the *in vivo* and *in vitro* results demonstrate that *C. parvum* infection is associated with downregulation of H3K27me3 and H3K36me3 in the infected intestinal epithelial cells. Further, we performed western blotting analysis to determine which stages of the parasite could affect the host methylation events during *C. parvum* infection *in vitro*. At 24h PI, when there is predominant existence of asexual stages of the parasite, H3K36me3 was observed to be downregulated by 0.5-fold in infected HCT8 cells. Whereas, H3K27me3 was relatively downregulated by 0.5-fold in infected cells when sexual forms of the parasite are predominant (Figure 7E, F).

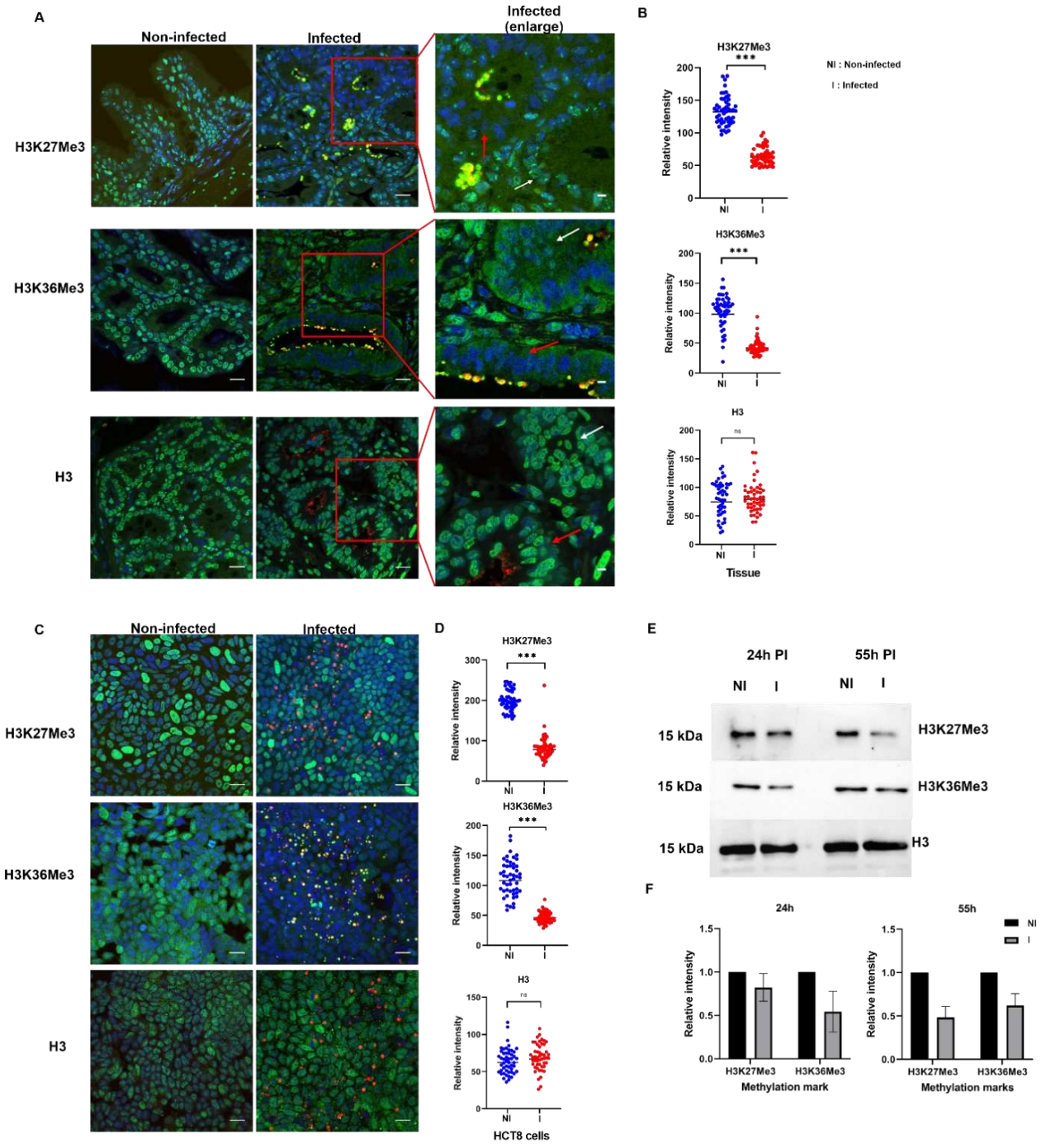


Figure 7. Effect of *C. parvum* infection on host histone methylation events. (A). Immunofluorescence analysis of histone methylation events during *C. parvum* infection *in vivo* at day 60 PI. The red squares represent enlarged images of the infected tissue. Red and white arrows represent infected and uninfected epithelial cells within the intestinal crypt, respectively. Co-staining with anti- histone methylation antibodies such as H3K36me3, H3K27me3, Histone H3 (green), anti-*Cryptosporidium* antibody (red) and DAPI (blue) of the ileo-caecal region of *C. parvum* infected SCID mice. (B). Quantification of fluorescence intensity signals of anti-methylation antibodies in *C. parvum* infected vs non-infected ileo-cecal tissue. The fluorescence signals were measured for 50 nuclei per sample. For the statistical

analysis, a mixed regression model was created considering fluorescence quantification as the main outcome and sample identifier as the random effect. The difference in the fluorescence intensity signals was statistically significant ($p<0.05$). (C). Immunofluorescence analysis of histone methylation events during *C. parvum* infection *in vitro* at 55h PI. (D). Quantification of fluorescence intensity signals of anti-methylation antibodies in *C. parvum* infected vs non-infected HCT8 cells. The fluorescence signals were measured for 50 nuclei per sample. The difference in the fluorescence intensity signals was statistically significant ($p<0.05$). (E). Western blotting analysis of histone methylation events during *C. parvum* infection *in vitro* at 24 h PI (asexual stage), 55 h PI (sexual stages) after purification of histones from host cell. F) The histograms represent relative intensity signals measured in infected HCT-8 cells with respect to non-infected HCT-8 cells. Each sample was normalized to the H3 used as internal control. The graph represents means in triplicate values. NI – Non-infected HCT8 cells. I – Infected HCT8 cells. Scale bar – 20 μm. Results are representative of three independent experiments.

Discussion

We investigated the potential significance of histone lysine methylation and methyltransferases during *C. parvum* infection. This is the first study to comprehensively identify KMTs in the *C. parvum* genome and to characterize lysine methylation events on host and parasite histones following infection, thereby recognizing the importance of epigenetics in the development and potential pathogenicity of *Cryptosporidium*.

Dozens of proteins capable of methylating specific residues have been characterized in higher eukaryotes, and all but one of these enzymes possess a SET-domain. Our *in silico* analysis using InterPro database identified 10 SET-domain containing proteins in *C. parvum* which we names CpSET proteins. Our sequence analysis emphasized their conservation in other sequenced *Cryptosporidium* genomes, suggesting the parasite ancestor had acquired these genes before speciation and divergence within this genus. However, KMTs belonging to the DOT1 family were detected in *C. muris* and *C. andersoni,* but not in *C. parvum*. The absence of DOT1 domain containing lysine methyltransferases in some *Cryptosporidium* species (as in related genera including *Toxoplasma* [43] and *Plasmodium* [28] suggests a secondary loss of the corresponding genes during the evolution of the phylum Apicomplexa. Along with the SET domains, the presence of PHD zinc finger domain [44] and bromodomain [45] suggests that the CpSETs proteins may form protein complexes and interact with chromatin. The PHD zinc finger domains and bromodomains could facilitate binding of the CpSET proteins to chromatin and assist in lysine methylation of histones catalyzed by the SET domain.

Results

Some histone modifications catalyzed by KMTs possess narrow substrate specificities, often targeting a single lysine within the respective substrates. KMTs also differ in their preference for different methylation states (mono-, di-, or tri- methylation) of lysine residues. In spite of the conserved overall structural plasticity, the variations at the active sites were shown to contribute to their varying substrate specificities [46]. We aligned the primary sequence of the SET-domain of identified CpSET proteins with different families of known KMTs. The SET domain consists of four signature motifs; motif I to IV belong to the pre-SET region of the SET domain, out of which the motifs III and IV are highly conserved and form a pseudoknot structure. The structural significance of this knot is to bring two conserved motifs together to form an active site immediately close to the motif I which is a binding site for the methyl donor S-adenosylmethionine (AdoMet). Thus, identification of these catalytically essential motifs involved in transfer of methyl group in 8 out of 10 putative CpSETs encouraged us to speculate that *C. parvum* KMTs are functionally active.

Out of all the putative parasite methyltransferases, CpSET1, CpSET2 and CpSET8 were identified as potential histone KMTs. All the residues of the signature motifs of SET and post-SET domains were conserved in the CpSET1 primary amino acid sequence. Moreover, CpSET1 clustered together with other SET1 family homologues with high bootstrap support in the present phylogenetic analysis. ScSET1, one of the homologues grouping together with apicomplexan enzymes associated with tri-methylation of H3K4 in the regions of genes which are transcribed early and is considered a mark of transcription activation [47]. Moreover, the domain organization of CpSET1 consists of bromo- and PHD domains which could interact with trimethylated H3K4 [48], suggesting that CpSET1 employs these interacting domains to target euchromatin and methylate H3K4. In addition, superimposition of CpSET1 3-D model with the available crystal structure of human MLL1, one of the members of the SET1 family, strongly suggested that CpSET1 is a structurally active HKMT. MLL1 was shown to catalyze multiple methylations. The superimposition analysis shows that CpSET1 conserves all the active site residues (Phe 2159, Tyr 2217, Tyr 2219, Phe 2221, Cys 2156 to Phe 2158) found in the SET-domain of MLL1. Moreover, the MLL1 SET-domain has similar arrangement to other SET domains [49]. CpSET1 also has conserved key tyrosine residues (Tyr 2129 Tyr 2217) required for the transfer of the methyl group. Even though, sequence analysis indicates that these tyrosine residues constrain HKMTs such as SET8 to be mono-methylases. It can be speculated that certain modulations in the configuration of the active site of CpSET1 could allow multiple methylation events which is the case for MLL1. Superposed structure of MLL1

SET domain with other HKMTs (SET7/9, SET8 and Dim5) revealed that MLL1 has a more spacious active site [49]. This spacious active site is attributed to a shift in the orientation of SET-I region and C-terminal flanking region in MLL1. This feature is also evident in CpSET1 after superimposition with MLL1. The CpSET1 residues (Cys 2156 to Phe 2158) are conserved which could be responsible for this change in orientation and allowing free movement of the lysine side chain. Thus, CpSET1 can be predicted to mono, di or tri-methylate H3K4. Interestingly, MLL family members (MLL1-4, SET1A and SET1B) are known to methylate H3K4 and have pivotal roles in the regulation of the transcription of genes involved in development, hematopoiesis [49], and cell cycle progression [50]. In this context, the immunofluorescence labeling observed during the development of the parasite using anti-H3K4me3 antibody could be attributed to the existence of a functional CpSET1 representing a MLL1 member of the HKMT family *Cryptosporidium*. Moreover, the relatively high expression of *CpSET1* gene during parasite development suggests that H3K4 methylation is not a dynamic, but a rather stable post-translational, modification necessary during all the stages of the parasite life cycle. In addition, H3K4me3 marks the promoter of actively transcribed genes in different apicomplexan parasites such as *T. gondii* [51], *T. annulata* [27] and *P. falciparum* [25]. Hence, the stable nature of this mark can be attributed to the fact that this modification is involved in expression of actively transcribing genes during each stage of the parasite. However, the open conformation of the SET domain of MLL1 has been predicted to be not efficient to transfer the methyl group from the cofactor S-adenosyl-L-methionine (AdoMet) to the target lysine [49]. Recently, other components such as RBBP5-ASH2L were shown to bind and activate MLL family methyltransferases through a conserved mechanism [52]. As the SET domain of CpSET1 is structurally identical to MLL1 SET domain, the involvement of other components needed by CpSET1 to maintain its HKMT activity could be investigated in further studies. Even though, we made preliminary attempts to clone and purify the CpSET1 domain in a bacterial system, Western blots analysis did not show detectable amounts of CpSET1 in the soluble fraction after induction and lysis of bacteria. Further studies should work on standardizing the solubility protocols for the concerned domain.

CpSET2 and CpSET8 were the other two HKMTs of *Cryptosporidium* identified to be structurally active. Even though, the phylogenetic tree reconstructed in the present study do not strongly support the grouping of CpSET2 with homologous enzymes from Apicomplexa and others eukaryotes, analysis of its primary sequence revealed the presence of a typical post-SET domain and a cysteine rich N-terminal region preceding the SET domain which both represent

peculiarities of proteins belonging to the SET2 family of HKMTs [53]. Moreover, the superimposition of 3D homology models of the SET domain of CpSET2 with a template SET domain of SETD2 (protein data bank: 6J9J.A) revealed 43 % identity between the two structures. Interestingly, SETD2, is the only HKMT known to mediate tri-methylation of H3K36, whereas other H3K36 methyltransferases can only mono or di-methylate H3.

Strikingly, H3K36me3 is known to be mediated by a single HKMT such as SETD2, whereas other H3K36 methyltransferases can only mono- and di-methylate H3K36 [54]. Intriguingly, mutating arginine residue (Arg1625Cys) present in the SET domain results in an enzymatically inactive SETD2 failing to tri-methylate H3K36 [55]. This arginine residue (Arg 648) is conserved in CpSET2. Thus, based on the structurally conserved residue it can be predicted to tri-methylate H3K36 in *Cryptosporidium*. In addition, the use of anti-H3K36me3 antibody, detected this methylation mark to be distributed throughout the life cycle stages of the parasite.. H3K36me3 has been reported to be a mark of transcriptional elongation in higher eukaryotes [56], but methylation of H3K36 was linked to repression of *var* genes in *Plasmodium* parasites [57]. Thus, the significance of this methylation mark in *Cryptosporidium* remains to be explored.

The SET8 family of HKMTs were characterized to mono-methylate H4 in humans [58]. The identification and characterization of SET8-related homologs in Apicomplexa, such as *Plasmodium* [28] and *Toxoplasma* [24], overruled the thought that this enzyme is restricted to metazoans [46]. CpSET8 identified in our study was also predicted to methylate H4K20 according to our phylogenetic analysis. Thus, reinforcing the importance of histone methylations in chromatin structure and function in Apicomplexa. The superimposition of 3D homology model of SET domain of CpSET8 with a template SET domain of SET8 (protein data bank: 5teg.A) revealed 44 % identity between the two structures. The conserved tyrosine residues (Tyr 245 and Tyr 334) within the active site of human SET8 are responsible for maintaining an intricate network of hydrogen bonds to position the side chain of only mono-methylated lysine residue [58]. On the contrary, Dim-5, a HKMT which can tri-methylate its target lysine can accommodate mono-, di- and tri-methylated lysine in its active site. This characteristic of Dim-5 has been attributed to the replacement of one of the tyrosine residues to phenylalanine residue (Tyr 178 and Phe 281) [59]. Interestingly, structural alignment revealed that in CpSET8 as well one of these tyrosine residues is replaced by phenylalanine residue (Tyr 445 and Phe 537). Thus, suggesting that CpSET8 is capable of adding multiple methyl group to its target lysine.

Thus, based on the analysis of structural elements, we hypothesize that CpSET8 might methylate H4K20me1, H4K20me2 and H4K20me3 like SET8 of *T gondii* [24]. This structural analysis was confirmed herein by showcasing HKMT activity of CpSET8 domain for H4K20me3. Along with the suspected dynamic nature of CpSET8, the methylation events predicted for this HKMT were observed to be regulated during parasite differentiation. This phenomenon can be considered as a characteristic of apicomplexan parasites, as H4K20me3 marks in *T. gondii* were reported to be cell-cycle dependent [24]. The possibility that CpSET8 methylates other targets cannot be excluded. However, unlike other HKMTs such as SMYD3 which are known to have multiple targets [59], [60], the SET8 family enzymes have only been shown to have a single target, i.e. H4K20 [53]. We propose that CpSET8 is capable of methylating H4K20 and further studies mutating the predicted catalytic residues of the whole protein could reveal the function of the SET domain

CpAKMT was another identified KMT which differed in the C terminal region wherein it retains two extra cysteines in addition to the post-SET domain. Phylogenetic analysis revealed that CpAKMT and its homologues from other Apicomplexa clustered together and represented a sister-group of HsSMYD3, as described in previous evolutionary studies including *Plasmodium* [28] and *Toxoplasma* KMTs [61]. The lack of MYND zinc finger domain, a fundamental feature of SMYDs, allows to group the AKMT homologues as a distinct family of KMTs. Structural comparison between the AKMT of *T. gondii* and SMYD proteins revealed that SMYD1-3 do not seem to possess the necessary features that are compatible for dimer formation whereas AKMT are dimeric enzymes [61]. In *T. gondii*, a functional AKMT was reported to be localized at the apical complex and associated with parasite motility and egress [62]. Moreover, lack of a MYND domain in AKMT might correlate with its specific function outside the nucleus [63]. Interestingly, immunofluorescence observations detected the labeling of histone lysine methylations at the apical region of *C. parvum* sporozoites and merozoites. In addition, the *CpAKMT* gene had a relatively high expression during the merozoite development and egress in *C. parvum*. In *P. falciparum,* histones released from the parasite exert a disruptive effect on the endothelial barrier function through a charge-related mechanism in order to induce pro-inflammatory responses [64]. Along with the extra-nuclear localization of histones, histone modification such as H3K9me1 in *P. falciparum* has been recognized as a new function linked to the parasitophorous vacuole and the interaction of the parasite with the host [65]. It is possible that CpAKMT is a functional KMT localized at the apical region capable of methylating extra-nuclear histones of *C. parvum* or non-histone proteins at the apical region to

assist in motility. Similar to SMYD3 which was recently characterized as a non-histone methyltransferase, considering its cytoplasmic localization [66]. Further studies have to be carried out to address this functional aspect.

KMTox was another identified KMT in *C. parvum*. First identified in *T. gondii*, the presence of High Mobility Group (HMG) domain in KMTox allows it to recognize the bent DNA with the SET domain involved in the methylation of histones H4 and H2A *in vitro* [67]. Even if the HMG domain was not identified in CpKMTox, the phylogenetic analysis based on SET-domain sequences clustered together all apicomplexan KMTox with a high bootstrap value suggesting that CpKMTox could be predicted as a novel histone H4- and H2A-specific methyltransferase. Even though it was previously reported that TgKMTox formed a distinct clade with no obvious homologues in the Apicomplexa lineage [67], our phylogenetic analysis identified other apicomplexan parasites retaining KMTox, such as *C. parvum* and *B. besnoiti*, that could represent a new clade of KMTs only found in this group of protozoa.

The nine cysteines residues of the pre-SET domain usually found in the SUV39 family of KMTs[68] were not identified in CpSETs. In parallel, none of the CpSETs clustered in our phylogenetic tree with the known homologues belonging to this family as previously reported [24]. Thus, it can be speculated that either H3K9 methylation is not a crucial post-translational modification required for the survival of *C. parvum* or that a yet unknown CpSET may carry out this function. The post-SET region of KMTs also consists of conserved cysteine residues ($CXCX_4C$). These three cysteines coordinate a zinc ion tetrahedrally together with cysteine of motif III of SET domain to form a narrow channel to accommodate the target lysine side chain. Consequently, the post-SET region is extremely crucial for the activity of a functional KMT. We observed that in CpSET1, CpSET2, CpSET Unk2 and CpSET Unk3 the cysteines of the post-SET region along with cysteine of motif III were conserved. However, CpSET Unk2 and CpSET Unk3 lacked the presence of the signature of motif I and could not be grouped together with known families of KMTs. On the other hand, CpSET Unk1 presented a variant of post-SET domain which is observed in the SMYD or SUV4-20 families of KMTs. The presence of a MYND zinc finger motif in CpSET Unk1 and its weak association with *H. sapiens* SMYD3 in our phylogenetic tree suggests that this CpSET could belong to the SMYD subfamily, which is composed of H3K4-specific methylase [59]. However, due to their high variability in terms of primary sequence, the functions associated with these CpSETs cannot be affirmatively predicted and thus these enzymes were considered as unknowns. Unlike the other characterized KMTs based on phylogenetic and structural analysis which show dynamic expression during

the intracellular stages of the parasite, *CpSET Unk1*, *CpSET Unk 2* and *CpSET Unk 3* expression levels were relatively unaffected. Thus, these putative KMTs may not play a role in parasite development.

To complete this overview, we could not identify any KDMs containing JmjC-domain in *Cryptosporidium*, highlighting that mechanism for histone demethylation is not present in this parasite as reported in previous studies [28], [69].

Strikingly, we provide new insights into the effect of *C. parvum* infection on the host histone lysine methylation events. Indeed, the modulation of different lysine methylation marks in *C. parvum* infected mice was observed. Indeed, the modulation of two distinct lysine methylation marks in *C. parvum* infected mice and HCT8 cells was described with the massive loss in the methylation marks of H3K36me3 and H3K27me3 in the presence of the parasite. Polycomb repressive complex 2 (PRC2) is a well-conserved epigenetic regulator, responsible for H3K27 methylation. EZH2 is the functional subunit of PRC2 which are responsible for the histone methylation at H3K27 [70]. PRC2 is essential for normal development, and deregulation of its function is associated with diverse phenotypes in development and disease, especially cancer. For example, the PRC paralogs EZH2 and EZH1 are universally upregulated and downregulated in multiple cancers, respectively [71]. Similarly, H3K36me3 is known to play a crucial role in wide range of biological processes such as transcriptional fidelity, mRNA splicing and DNA damage repair by marking the body of actively transcribing genes. In addition to the deregulation of regulatory factors responsible for H3K36 methylation which gives rise to developmental disorders and cancer, mutations in H3K36 are also detected in human tumors [72]. Moreover, H3K36 trimethylation was reported to antagonize PRC2-mediated H3K27 methylation [73]. Our *in vivo,* as well as *in vitro,* results strikingly demonstrated downregulation of both H3K36me3 and H3K27me3 marks upon *C. parvum* infection. In addition, H3K27me3 was shown to be downregulated during the development of asexual stage of the parasite *in vitro*. Thus, it can be speculated that *C. parvum* during its asexual development is capable of transferring virulence factors into the host cell which in turn inhibits the PRC2 complex and downregulates H3K27me3. *C. parvum* was reported to secrete effector molecules in the form of non-coding RNA (ncRNA) to induce transcriptional suppression of host genes by upregulation of the H3K9me3 mark [42]. These RNA transcripts bind to G9a, a histone methyltransferase, and recruit it to the promoter regions for H3K9 methylation. Interestingly, *T. annulata*, a parasite with oncogenic potential, upon infection induces host SMYD3 expression to catalyze H3K4 methylation at matrix metalloproteinases 9

(MMP-9) promoter region and induce *MMP-9* transcription [43]. MMPs are known to mediate the metastatic phenotypes of *T. annulata*-transformed cells [74].

Similarly, the neoplasia developed as a result of *C. parvum* infection can be speculated to be regulated by massive loss in the methylation marks such as H3K36me3 and H3K27me3. Interestingly, SETD2, an epigenetic regulator of H3K36 methylation, was established as a tumor suppressor gene in CRC and loss of this methyltransferase augments WNT/β-catenin signaling pathway and aggressive tumor development [75]. Moreover, oncogenic Epstein-Barr virus (EBV) encodes an oncoprotein called latent membrane protein (LMP1) which can induce H3K27me3 demethylation through activation of histone demethylase, KDM6B [76]. In addition, LMP1 was also reported to target EZH2 and reduce H3K27me3 [77]. Similar strategy can be expected to be exploited by *C. parvum*. The PI3K/AKT signaling pathway known to be activated during *Cryptosporidium* infection [78] can downregulate H3K27 and H3K4 methylations and consequently activate Epithelial Mesenchymal Transition (EMT) in gastric cancers [79]. This result is consistent with a recent microarray study reporting that EMT takes place within the tumor microenvironment induced by *C. parvum* infection in a rodent model [41]. Thus, it can be speculated that the parasite is capable of modulating EMT associated genes through epigenetic mechanisms. Further studies are required to validate this aspect.

In conclusion, lysine methylases were successfully characterized in *C. parvum.* Since these enzymes are involved in the development of the parasite life cycle, they may be considered as potential targets to curb infection, opening new avenues for anti-parasite drug discovery. On the other hand, our work contributes to the growing evidence suggesting that protozoan parasites are able to manipulate host cells via epigenetic modifications of the host genome altering transcription and signaling pathways. This unexplored territory of epigenetic modulations in *C. parvum* infection would require further investigation for instance by ChIP-sequencing to identify all regulated genes.

Acknowledgments:

We wish to thank the Bioimaging Center Lille, Institut Pasteur de Lille, France for access to the microscopy instruments.

Disclosure statement

No potential conflict of interest was reported by the authors

Results

Data availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials

Funding:

This study was supported by the Plan Cancer "Epigénétique et cancer" 2015 (PARA-CAN #PARA-15-RCA), the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Institute Pasteur of Lille, the University of Lille and the Centre Hospitalier Regional Universitaire (CHRU) de Lille. M.S. was supported by a PhD fellowship from the University of Lille. R.W. is a Research Associate with the Belgian National Funds for Scientific Research (FRS-FNRS).

References

[1] N. Arisue and T. Hashimoto, "Phylogeny and evolution of apicoplasts and apicomplexan parasites," *Parasitol. Int.*, vol. 64, no. 3, pp. 254–259, Jun. 2015.

[2] N. E. Ramirez, L. A. Ward, and S. Sreevatsan, "A review of the biology and epidemiology of cryptosporidiosis in humans and animals.," *Microbes Infect.*, vol. 6, no. 8, pp. 773–85, Jul. 2004.

[3] I. A. Khalil *et al.*, "Morbidity, mortality, and long-term consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyzes study," *Lancet Glob. Heal.*, vol. 6, no. 7, pp. e758–e768, Jul. 2018.

[4] K. L. Kotloff *et al.*, "Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study," *Lancet*, vol. 382, no. 9888, pp. 209–222, Jul. 2013.

[5] D. G. Korich, J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling, "Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability," *Appl. Environ. Microbiol.*, vol. 56, no. 5, pp. 1423–1428, 1990.

[6] S. Baldursson and P. Karanis, "Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2004-2010.," *Water Res.*, vol. 45, no. 20, pp. 6603–14, Dec. 2011.

[7] A. Efstratiou, J. E. Ongerth, and P. Karanis, "Waterborne transmission of protozoan parasites: Review of worldwide outbreaks - An update 2011–2016," *Water Res.*, vol. 114, pp. 14–22, May 2017.

[8] W. Checkley *et al.*, "A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*," *Lancet Infect. Dis.*, Sep. 2014.

[9] P. Xu *et al.*, "The genome of *Cryptosporidium hominis*," *Nature*, vol. 431, no. 7012, pp. 1107–12, Oct. 2004.

[10] M. S. Abrahamsen *et al.*, "Complete genome sequence of the apicomplexan, *Cryptosporidium parvum*," *Science*, vol. 304, no. 5669, pp. 441–445, Apr. 2004.

[11] L. V. S. Matos, J. McEvoy, S. Tzipori, K. D. S. Bresciani, and G. Widmer, "The transcriptome of *Cryptosporidium* oocysts and intracellular stages," *Sci. Rep.*, vol. 9, no. 1, Dec. 2019.

[12] C. Lippuner *et al.*, "RNA-Seq analysis during the life cycle of *Cryptosporidium parvum* reveals significant differential gene expression between proliferating stages in the intestine and infectious sporozoites," *Int. J. Parasitol.*, vol. 48, no. 6, pp. 413–422, May 2018.

[13] S. Vinayak *et al.*, "Genetic modification of the diarrhoeal pathogen *Cryptosporidium parvum*," *Nature*, vol. 523, no. 7561, pp. 477–480, Jul. 2015.

[14] J. Tandel *et al.*, "Life cycle progression and sexual development of the apicomplexan parasite *Cryptosporidium parvum*," *Nat. Microbiol.*, vol. 4, no. 12, pp. 2226–2236, Dec. 2019.

[15] T. J. Templeton *et al.*, "Comparative analysis of apicomplexa and genomic diversity in eukaryotes," *Genome Res.*, vol. 14, no. 9, pp. 1686–1695, Sep. 2004.

[16] M. A. Hakimi and K. W. Deitsch, "Epigenetics in Apicomplexa: control of gene expression during cell cycle progression, differentiation and antigenic variation," *Curr. Opin. Microbiol.*, vol. 10, no. 4, pp. 357–362, Aug. 2007.

[17] S. Kumar, V. Chinnusamy, and T. Mohapatra, "Epigenetics of Modified DNA Bases: 5-Methylcytosine and Beyond," *Front. Genet.*, vol. 9, Dec. 2018.

[18] D. Husmann and O. Gozani, "Histone lysine methyltransferases in biology and disease," *Nat. Struct. Mol. Biol.*, vol. 26, no. 10, pp. 880–889, Oct. 2019.

[19] C. E. Barnes, D. M. English, and S. M. Cowley, "Acetylation & Co: an expanding repertoire of histone acylations regulates chromatin and transcription," *Essays Biochem.*, vol. 63, no. 1, pp. 97–107, 2019.

[20] M. Gissot, S. W. Choi, R. F. Thompson, J. M. Greally, and K. Kim, "*Toxoplasma gondii* and *Cryptosporidium parvum* lack detectable DNA cytosine methylation," *Eukaryot. Cell*, vol. 7, no. 3, pp. 537–540, Mar. 2008.

[21] L. Vanagas, V. Jeffers, S. S. Bogado, M. C. Dalmasso, W. J. Sullivan, and S. O. Angel,

"*Toxoplasma* histone acetylation remodelers as novel drug targets," *Expert Rev. Anti. Infect. Ther.*, vol. 10, no. 10, pp. 1189–1201, Oct. 2012.

[22] M. J. Chua *et al.*, "Effect of clinically approved HDAC inhibitors on *Plasmodium*, Leishmania and Schistosoma parasite growth," *Int. J. Parasitol. Drugs drug Resist.*, vol. 7, no. 1, pp. 42–50, Apr. 2017.

[23] F. Guo, H. Zhang, N. N. McNair, J. R. Mead, and G. Zhu, "The Existing Drug Vorinostat as a New Lead Against Cryptosporidiosis by Targeting the Parasite Histone Deacetylases," *J. Infect. Dis.*, vol. 217, no. 7, pp. 1110–1117, Mar. 2018.

[24] C. F. Sautel *et al.*, "SET8-mediated methylations of histone H4 lysine 20 mark silent heterochromatic domains in apicomplexan genomes," *Mol. Cell. Biol.*, vol. 27, no. 16, pp. 5711–5724, Aug. 2007.

[25] J. J. Lopez-Rubio, A. M. Gontijo, M. C. Nunes, N. Issar, R. Hernandez Rivas, and A. Scherf, "5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites," *Mol. Microbiol.*, vol. 66, no. 6, pp. 1296–1305, Dec. 2007.

[26] T. Chookajorn *et al.*, "Epigenetic memory at malaria virulence genes," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 3, pp. 899–902, Jan. 2007.

[27] K. Cheeseman *et al.*, "Dynamic methylation of histone H3K18 in differentiating *Theileria* parasites," *Nat. Commun.*, vol. 12, no. 1, Dec. 2021.

[28] L. Cui, Q. Fan, L. Cui, and J. Miao, "Histone lysine methyltransferases and demethylases in *Plasmodium falciparum*," *Int. J. Parasitol.*, vol. 38, no. 10, pp. 1083–1097, Aug. 2008.

[29] N. Saksouk *et al.*, "Histone-modifying complexes regulate gene expression pertinent to the differentiation of the protozoan parasite *Toxoplasma gondii*," *Mol. Cell. Biol.*, vol. 25, no. 23, pp. 10301–10314, Dec. 2005.

[30] M. Villares, J. Berthelet, and J. B. Weitzman, "The clever strategies used by intracellular parasites to hijack host gene expression," *Semin. Immunopathol.*, vol. 42, no. 2, pp. 215–226, Apr. 2020.

[31] A. M. Cock-Rada *et al.*, "SMYD3 promotes cancer invasion by epigenetic upregulation of the metalloproteinase MMP-9," *Cancer Res.*, vol. 72, no. 3, pp. 810–820, Feb. 2012.

[32] M. Sawant *et al.*, "*Cryptosporidium* and Colon Cancer: Cause or Consequence?," *Microorganisms*, vol. 8, no. 11, pp. 1–15, Nov. 2020.

[33] S. Kumar, G. Stecher, M. Li, C. Knyaz, and K. Tamura, "MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms," *Mol. Biol. Evol.*, vol. 35,

no. 6, pp. 1547–1549, Jun. 2018.

[34] C. J. Williams *et al.*, "MolProbity: More and better reference data for improved all-atom structure validation," *Protein Sci.*, vol. 27, no. 1, pp. 293–315, Jan. 2018.

[35] V. B. Chen *et al.*, "MolProbity: all-atom structure validation for macromolecular crystallography," *Acta Crystallogr. D. Biol. Crystallogr.*, vol. 66, no. Pt 1, pp. 12–21, 2010.

[36] L. Holm, "Using Dali for Protein Structure Comparison," *Methods Mol. Biol.*, vol. 2112, pp. 29–42, 2020.

[37] E. F. Pettersen *et al.*, "UCSF ChimeraX: Structure visualization for researchers, educators, and developers," *Protein Sci.*, vol. 30, no. 1, pp. 70–82, Jan. 2021.

[38] A. Dibao-Dina, J. Follet, M. Ibrahim, A. Vlandas, and V. Senez, "Electrical impedance sensor for quantitative monitoring of infection processes on HCT-8 cells by the waterborne parasite *Cryptosporidium*," *Biosens. Bioelectron.*, vol. 66, pp. 69–76, Apr. 2015.

[39] A. R. Keegan, S. Fanok, P. T. Monis, and C. P. Saint, "Cell culture-Taqman PCR assay for evaluation of *Cryptosporidium parvum* disinfection," *Appl. Environ. Microbiol.*, vol. 69, no. 5, pp. 2505–2511, May 2003.

[40] G. Certad *et al.*, "*Cryptosporidium parvum*, a potential cause of colic adenocarcinoma," *Infect. Agent. Cancer*, vol. 2, no. 1, 2007.

[41] M. Sawant *et al.*, "Persistent *Cryptosporidium parvum* Infection Leads to the Development of the Tumor Microenvironment in an Experimental Mouse Model: Results of a Microarray Approach," *Microorganisms*, vol. 9, no. 12, Dec. 2021.

[42] Y. Wang, A. Y. Gong, S. Ma, X. Chen, J. K. Strauss-Soukup, and X. M. Chen, "Delivery of parasite Cdg7_Flc_0990 RNA transcript into intestinal epithelial cells during *Cryptosporidium parvum* infection suppresses host cell gene transcription through epigenetic mechanisms," *Cell. Microbiol.*, vol. 19, no. 11, Nov. 2017.

[43] S. E. Dixon, K. L. Stilger, E. V. Elias, A. Naguleswaran, and W. J. Sullivan, "A decade of epigenetic research in *Toxoplasma gondii*," *Mol. Biochem. Parasitol.*, vol. 173, no. 1, pp. 1–9, Sep. 2010.

[44] K. Jain *et al.*, "Characterization of the plant homeodomain (PHD) reader family for their histone tail interactions," *Epigenetics Chromatin*, vol. 13, no. 1, Jan. 2020.

[45] F. Winston and C. David Allis, "The bromodomain: a chromatin-targeting module?," *Nat. Struct. Biol.*, vol. 6, no. 7, pp. 601–604, 1999.

[46] K. Nishioka *et al.*, "PR-Set7 is a nucleosome-specific methyltransferase that modifies

lysine 20 of histone H4 and is associated with silent chromatin," *Mol. Cell*, vol. 9, no. 6, pp. 1201–1213, 2002.

[47] H. Santos-Rosa *et al.*, "Active genes are tri-methylated at K4 of histone H3," *Nature*, vol. 419, no. 6905, pp. 407–411, Sep. 2002.

[48] Y. Zhang, "It takes a PHD to interpret histone methylation," *Nat. Struct. Mol. Biol.*, vol. 13, no. 7, pp. 572–574, Jul. 2006.

[49] S. M. Southall, P. S. Wong, Z. Odho, S. M. Roe, and J. R. Wilson, "Structural basis for the requirement of additional factors for MLL1 SET domain activity and recognition of epigenetic marks," *Mol. Cell*, vol. 33, no. 2, pp. 181–191, Jan. 2009.

[50] B. P. Mishra, K. I. Ansari, and S. S. Mandal, "Dynamic association of MLL1, H3K4 trimethylation with chromatin and Hox gene expression during the cell cycle," *FEBS J.*, vol. 276, no. 6, pp. 1629–1640, Mar. 2009.

[51] M. Gissot and K. Kim, "How epigenomics contributes to the understanding of gene regulation in *Toxoplasma gondii*," *J. Eukaryot. Microbiol.*, vol. 55, no. 6, pp. 476–480, Nov. 2008.

[52] Y. Li *et al.*, "Structural basis for activity regulation of MLL family methyltransferases," *Nature*, vol. 530, no. 7591, pp. 447–452, Feb. 2016.

[53] S. C. Dillon, X. Zhang, R. C. Trievel, and X. Cheng, "The SET-domain protein superfamily: protein lysine methyltransferases," *Genome Biol.*, vol. 6, no. 8, 2005.

[54] E. J. Wagner and P. B. Carpenter, "Understanding the language of Lys36 methylation at histone H3," *Nat. Rev. Mol. Cell Biol.*, vol. 13, no. 2, pp. 115–126, Feb. 2012.

[55] K. E. Hacker *et al.*, "Structure/Function Analysis of Recurrent Mutations in SETD2 Protein Reveals a Critical and Conserved Role for a SET Domain Residue in Maintaining Protein Stability and Histone H3 Lys-36 Trimethylation," *J. Biol. Chem.*, vol. 291, no. 40, pp. 21283–21295, Sep. 2016.

[56] R. J. Sims, K. Nishioka, and D. Reinberg, "Histone lysine methylation: a signature for chromatin function," *Trends Genet.*, vol. 19, no. 11, pp. 629–639, 2003.

[57] L. Jiang *et al.*, "PfSETvs methylation of histone H3K36 represses virulence genes in *Plasmodium* falciparum," *Nature*, vol. 499, no. 7457, pp. 223–227, 2013.

[58] B. Xiao *et al.*, "Specificity and mechanism of the histone methyltransferase Pr-Set7," *Genes Dev.*, vol. 19, no. 12, pp. 1444–1454, Jun. 2005.

[59] R. Hamamoto *et al.*, "SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells," *Nat. Cell Biol.*, vol. 6, no. 8, pp. 731–740, Aug. 2004.

[60] G. S. van Aller *et al.*, "Smyd3 regulates cancer cell phenotypes and catalyzes histone

H4 lysine 5 methylation," *Epigenetics*, vol. 7, no. 4, pp. 340–343, 2012.

[61] S. Sivagurunathan, A. Heaslip, J. Liu, and K. Hu, "Identification of functional modules of AKMT, a novel lysine methyltransferase regulating the motility of *Toxoplasma gondii*," *Mol. Biochem. Parasitol.*, vol. 189, no. 1–2, pp. 43–53, 2013.

[62] Y. Pivovarova *et al.*, "Structure of a Novel Dimeric SET Domain Methyltransferase that Regulates Cell Motility," *J. Mol. Biol.*, vol. 430, no. 21, pp. 4209–4229, Oct. 2018.

[63] A. T. Heaslip, M. Nishi, B. Stein, and K. Hu, "The motility of a human parasite, *Toxoplasma gondii*, is regulated by a novel lysine methyltransferase," *PLoS Pathog.*, vol. 7, no. 9, Sep. 2011.

[64] M. R. Gillrie *et al.*, "*Plasmodium falciparum* histones induce endothelial proinflammatory response and barrier dysfunction," *Am. J. Pathol.*, vol. 180, no. 3, pp. 1028–1039, 2012.

[65] Y. H. Luah, B. K. Chaal, E. Z. Ong, and Z. Bozdech, "A moonlighting function of *Plasmodium falciparum* histone 3, mono-methylated at lysine 9?," *PLoS One*, vol. 5, no. 4, 2010.

[66] D. Pereira, A. Richert, S. Medjkane, S. Hénon, and J. B. Weitzman, "Cell geometry and the cytoskeleton impact the nucleo-cytoplasmic localisation of the SMYD3 methyltransferase," *Sci. Rep.*, vol. 10, no. 1, Dec. 2020.

[67] C. F. Sautel *et al.*, "The histone methylase KMTox interacts with the redox-sensor peroxiredoxin-1 and targets genes involved in *Toxoplasma gondii* antioxidant defences," *Mol. Microbiol.*, vol. 71, no. 1, pp. 212–226, 2009.

[68] S. Rea *et al.*, "Regulation of chromatin structure by site-specific histone H3 methyltransferases," *Nature*, vol. 406, no. 6796, pp. 593–599, Aug. 2000.

[69] S. D. Rider and G. Zhu, "*Cryptosporidium*: genomic and biochemical features," *Exp. Parasitol.*, vol. 124, no. 1, pp. 2–9, Jan. 2010.

[70] A. Piunti and A. Shilatifard, "The roles of Polycomb repressive complexes in mammalian development and cancer," *Nat. Rev. Mol. Cell Biol.*, vol. 22, no. 5, pp. 326–345, May 2021.

[71] S. Wang *et al.*, "Polycomb group proteins in cancer: multifaceted functions and strategies for modulation," *NAR cancer*, vol. 3, no. 4, Oct. 2021.

[72] J. Li, J. H. Ahn, and G. G. Wang, "Understanding histone H3 lysine 36 methylation and its deregulation in disease," *Cell. Mol. Life Sci.*, vol. 76, no. 15, pp. 2899–2916, Aug. 2019.

[73] W. Yuan, M. Xu, C. Huang, N. Liu, S. Chen, and B. Zhu, "H3K36 methylation

antagonizes PRC2-mediated H3K27 methylation," *J. Biol. Chem.*, vol. 286, no. 10, pp. 7983–7989, Mar. 2011.

[74] R. E. Adamson and F. R. Hall, "Matrix metalloproteinases mediate the metastatic phenotype of *Theileria* annulata-transformed cells," *Parasitology*, vol. 113 (Pt 5), no. 5, pp. 449–455, 1996.

[75] H. Yuan *et al.*, "Histone methyltransferase SETD2 modulates alternative splicing to inhibit intestinal tumorigenesis," *J. Clin. Invest.*, vol. 127, no. 9, pp. 3375–3391, Sep. 2017.

[76] J. A. Anderton *et al.*, "The H3K27me3 demethylase, KDM6B, is induced by Epstein-Barr virus and over-expressed in Hodgkin's Lymphoma," *Oncogene*, vol. 30, no. 17, pp. 2037–2043, Apr. 2011.

[77] K. A. Martin, L. N. Lupey, and I. Tempera, "Epstein-Barr Virus Oncoprotein LMP1 Mediates Epigenetic Changes in Host Gene Expression through PARP1," *J. Virol.*, vol. 90, no. 19, pp. 8520–8530, Oct. 2016.

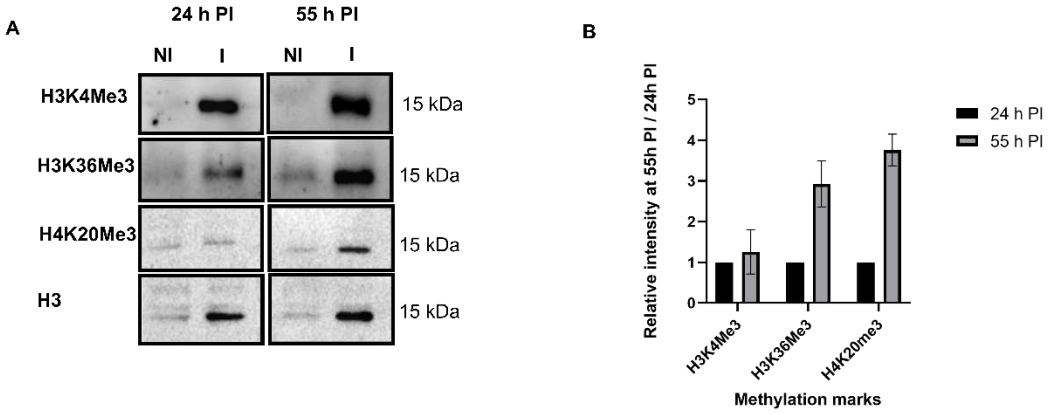
[78] J. R. Forney, D. B. DeWald, S. Yang, C. A. Speer, and M. C. Healey, "A role for host phosphoinositide 3-kinase and cytoskeletal remodeling during *Cryptosporidium parvum* infection," *Infect. Immun.*, vol. 67, no. 2, pp. 844–852, 1999.

[79] X. Liu *et al.*, "AURKA induces EMT by regulating histone modification through Wnt/β-catenin and PI3K/Akt signaling pathway in gastric cancer," *Oncotarget*, vol. 7, no. 22, pp. 33152–33164, May 2016.

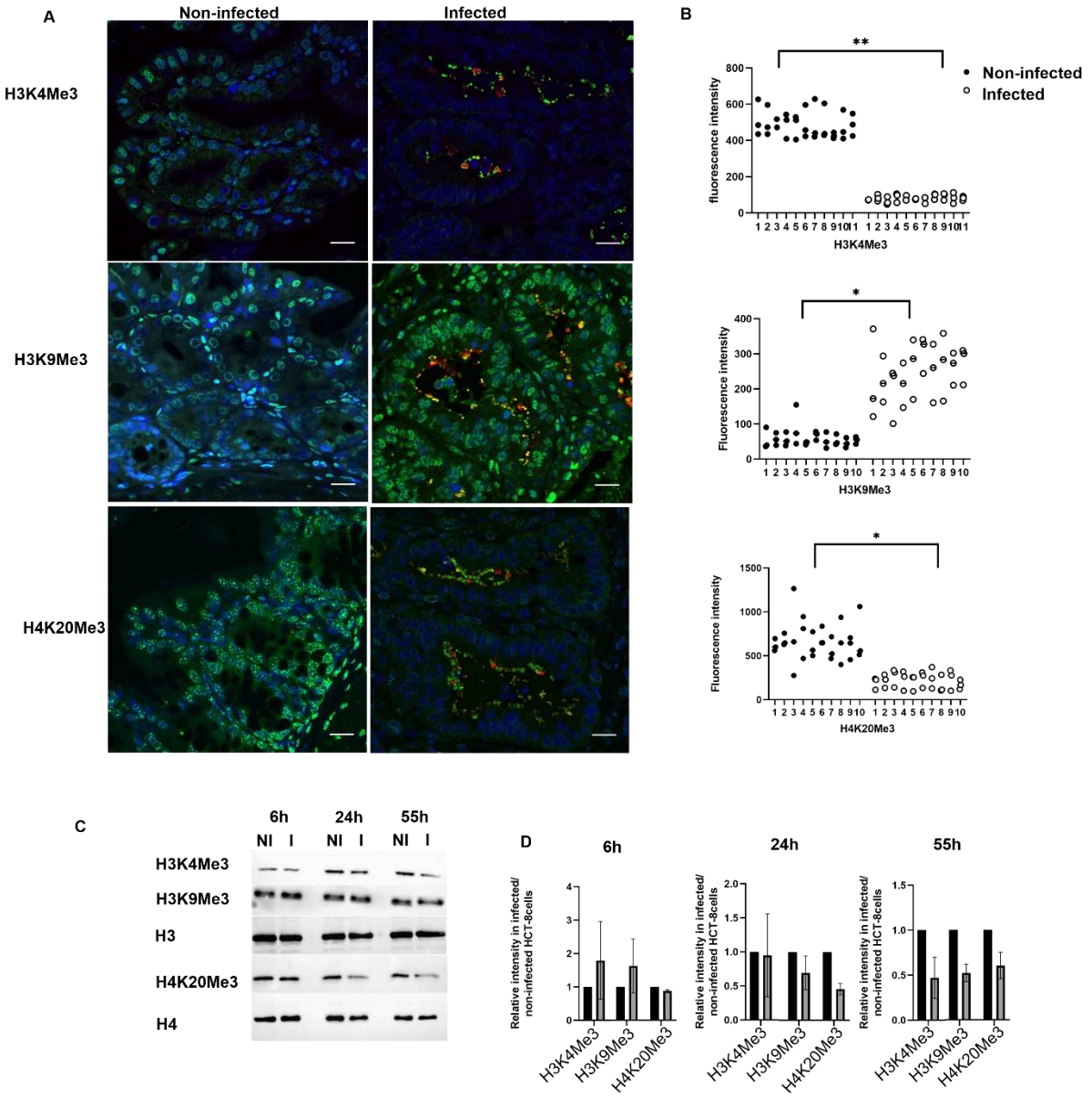
Results

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Hs H3.1         ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
Hs H3.2         ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
Hs H3.3         ARTKQTARKS TGGKAPRKQL ATKAARKSAP STGGVKKPHR YRPGTVALRE
Mm H3.1         ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
Mm H3.2         ARTKQTARKS TGGKAPRKQL ATKAARKSAP ATGGVKKPHR YRPGTVALRE
Mm H3.3         ARTKQTARKS TGGKAPRKQL ATKAARKSAP STGGVKKPHR YRPGTVALRE
Cp H3           ARTKQTARKS TGGKAPRKQL ASKGARKSAP VTGGVKKPRR YRPGTVALRE

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Hs H3.1         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
Hs H3.2         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEASEAYL
Hs H3.3         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSAAIG ALQEASEAYL
Mm H3.1         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEACEAYL
Mm H3.2         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSSAVM ALQEASEAYL
Mm H3.3         IRRYQKSTEL LIRKLPFQRL VREIAQDFKT DLRFQSAAIG ALQEASEAYL
Cp H3           IRRFQRSTEL LIRKLPFQRL VREIAQDFKT DLRFQSQAVM ALQEAAEAYL

                ....|....| ....|....| ....|....| ....|.
                        110        120        130
Hs H3.1         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Hs H3.2         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Hs H3.3         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Mm H3.1         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Mm H3.2         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Mm H3.3         VGLFEDTNLC AIHAKRVTIM PKDIQLARRI RGERA.
Cp H3           VGLFEDTNLC AIHAHRVTIM PKDIQLARRI RGER..

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
Hs H4           SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
Mm H4           SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISGLI
Cp H4           SGRGKGGKGL GKGGAKRHRK VLRDNIQGIT KPAIRRLARR GGVKRISALI

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
Hs H4           YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF
Mm H4           YEETRGVLKV FLENVIRDAV TYTEHAKRKT VTAMDVVYAL KRQGRTLYGF
Cp H4           YEEVRGVLKA FLETVIKDAV TYTEYARRKT VTAMDVVHAL KRQGKTLYGF

                ....|....|
                        110
Hs H4           GG........
Mm H4           GG........
Cp H4           GG........
```

Supplementary Figure 1. Multiple sequence alignment of histone proteins from homo sapiens, Mus musculus and *C. parvum.* Highly conserved lysine residues (K) are highlighted in red. The uniport accession numbers of the sequences used are Hs H3.1 (P68431), Hs H3.2 (Q71DI3), Hs H3.3 (P84243), Hs H4 (Q5CV68), Ms H3.1 (P84228), Ms H3.2 (P84244), Ms H3.3 (P68433), Ms H4 (P62806), Cp H3 (Q5CUJ9) and Cp H4 (Q5CV68)



Supplementary Figure 2. Western blot analysis of parasite histone methylation marks. (A). Western blotting analysis of histone lysine methylation modifications during *C. parvum* development *in vitro* at 24 h (asexual stages) and 55 h (sexual stages) PI after purification of histones from the parasites. (B). The histograms represent the relative intensity signals of methylation marks in the parasite at 55h PI relative to 24h PI. Each sample was normalized to the H3 used as internal control. The graph represents means in triplicate values. NI – Non-infected HCT-8 cells. I – Infected HCT-8 cells. Scale bar – 1 µm. Results are representative of three independent experiments.



Supplementary Figure 3. Effect of *C. parvum* infection on methylation marks *in vivo* and *in vitro*. (A). Immunofluorescence analysis of histone methylation events during *C. parvum* infection *in vivo* at day 60 PI. Co-staining with anti- histone methylation antibodies such as H3K4Me3, H3K9Me3, H4K20Me3 (green), anti-*Cryptosporidium* antibody (red) and DAPI (blue) of the ileo-caecal region of *C. parvum* infected SCID mice. (B). Quantification of fluorescence intensity signals of anti-methylation antibodies in *C. parvum* infected vs non-infected ileo-caecal tissue. The signal intensities were measured by nuclei. Numbers on the Y axis indicate individual nucleus. The black and white circles indicate uninfected and infected nuclei respectively. For the statistical analysis, a mixed regression model was created considering fluorescence quantification as the main outcomes and sample identifier as random effect. The difference in the fluorescence intensity signals was statistically significant ($p<0.05$). (C). Western blotting analysis of histone methylation events during *C. parvum* infection *in vitro* at 6h PI (trophozoite stage) 24 h PI (asexual stage), 55 h PI (sexual stages) after purification of

histones from host cell. (D). The histograms represent relative intensity signals measured in infected HCT-8 cells with respect to non-infected HCT-8 cells. Each sample was normalized to the H3 used as internal control. The graph represents mean of triplicate values. NI – Non-infected HCT8 cells. I – Infected HCT8 cells. Scale bar – 20 µm. Results are representative of three independent experiments.

Supplementary Table 1. Primary and secondary antibodies dilutions

Abbreviations: IHC: immunohistochemistry; ICC: Immunocytochemistry

Antibodies	**ICC/ IHC (dilutions)**	**Western blotting (dilutions)**
Anti-Histone H3 (tri methyl K4) antibody - ChIP Grade ab8580	1:1000	1:1000
Anti-Histone H3 (tri methyl K36) antibody - ChIP Grade ab9050	1:500	1:1000
Anti-Histone H3 (tri methyl K27) antibody - ChIP Grade [mAbcam 6002]	1:500	1:1000
Anti-Histone H4 (tri methyl K20) antibody [EPR17001(2)] - ChIP Grade	1:1000	1:1000
Anti-Histone H3 antibody - Nuclear Marker and ChIP Grade [ab1791]	1:100	1:1000
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)(ab150077)	1:1000	
A600FLR-20X Sporo-Glo™	1:50	
Goat Anti-Rabbit IgG H&L (HRP) (ab6721)		1:1000

Supplementary Table 2. Primers used for RT-qPCR analysis of putative KMTs

Gene ID	**Primers**	**Tm**	**Sequences**	**Fragment size (bp)**
cgd1-2170	F	60.1	5’ gctgaagcagtatcccgttgca 3’	80
	R	58.2	5’ tcgtctttcatacccagttcttgc 3’	
cgd4-370	F	58.4	5’ tgtgtaatcgccggatctc 3’	91
	R	59.7	5’ gctctttggcctcgttaagc 3’	
cgd4-2090	F	59.9	5’ gccagggaatttgggtttaacg 3’	84
	R	59.7	5’ ttcatcggttgcaatccctcc 3’	
cgd5-400	F	58.9	5’ gaaagatcctgcggagtatgc 3’	116
	R	58.5	5’ tcttcgagtccgacgca 3’	
cgd5-2340	F	58.2	5’ tgctaacgatggaagcgca 3’	84
	R	58.3	5’ gatctaccttctctttcgtcataccac 3’	
cgd6-1470	F	58.3	5’ gtagcttgcctagattggaaagca 3’	92
	R	59.5	5’ tggaataagtcctgttcctagctc 3’	
cdg7-5090	F	58.6	5’ agtgaatccacgacaaaaagctcc 3’	80
	R	58.4	5’ cccatccaagaatgcttgg 3’	
cgd8-2730	F	58.2	5’ tgacggtagaaagtgctagga 3’	116
	R	58.8	5’ cttgcttgatgaggaatgagagc 3’	
18S	F	58.2	5’ tgccttgaatactccagcatgg 3’	103
	R	59.6	5’ tacaaatgcccccaactgtcc 3’	

Results

3.2. Transcriptomic characterization of *C. parvum* induced digestive neoplasia.

This study is the subject of the article published in the journal "Microorganisms", 2021, *9* (12), 2569.

Title: **Persistent *Cryptosporidium parvum* infection leads to the development of tumor microenvironment in an experimental mouse model: Results of a microarray approach**

Manasi Sawant, Sadia Benamrouz-Vanneste, Anthony Mouray, Peggy Bouquet, Nausicaa Gantois, Colette Creusy, Erika Duval, Adriana Mihalache, Pierre Gosset, Magali Chabé, David Hot, Eric Viscogliosi and Gabriela Certad

My contribution to the study:

- Performing the experiments
- Analysis of the data
- Writing the manuscript

Overview :

C. parvum is an ubiquitous, intracellular parasite considered to be a major cause of diarrhea worldwide causing self-limited diarrhea in immunocompetent individuals and a life-threatening disease in those immunocompromised individuals. Moreover, growing epidemiological and experimental evidences have speculated a causal link between *C. parvum* infection and colon cancer.

The pathophysiological mechanisms of *Cryptosporidium* infection induced carcinogenesis are not completely understood and can be multifactorial. For instance, alterations in cellular expression of APC, p53, β-catenin and E-cadherin followed by prominent basolateral and cytoplasmic localization of β-catenin have suggested the involvement of a non-canonical Wnt pathway. In addition, the dilations of intercellular spaces and lateral membrane extensions in the ileo-caecal epithelia of mice infected with *C. parvum* was observed as one of the characteristic of the neoplasia induced by the parasite. In parallel, it is well known that inflammatory monocytes assist *C. parvum* to breach the epithelial barrier by delocalization of β-catenin and E-cadherin. Thus, it can be predicted that *C. parvum* infection which activates pro-inflammatory signaling pathway such as nuclear factor-kappa B (NF-κβ) to induce anti-apoptotic mechanisms can also transmit oncogenic signals to epithelial cells.

Interestingly, other cancer-associated signaling pathways such as Hedgehog and p38/MAPK are known to be altered by the parasite for its survival. However, with the limitation of these studies being carried out in already transformed intestinal epithelial cells, it was difficult to know if these mechanisms really play a significant role in *C. parvum* infection-induced cancer development.

With the transcriptomic approach, we aimed to decipher potential novel markers of *C. parvum* infection induced digestive cancer. The transcriptomes of *C. parvum* infected ileo-caecal regions of mice developing tumors were analyzed and compared to non-infected controls.

IPA analysis was performed to analyze the gene expression profile altered during *C. parvum* induced digestive neoplasia. We observed that *C. parvum* can resist the innate immune response generated by the host through an unknown mechanism. The innate immune response of the host was evident by the upregulated expression of IFNγ-stimulated genes. Members of the IFNγ induced GTPases superfamily are speculated to be the main targets of *C. parvum* to evade the innate immune response. The inflammatory response generated as a result of the persistent *C. parvum* infection eventually alters and downregulates the expression of anti-microbial peptides such as α-defensins. Molecular network analysis predicted IGF1 and TNFα mediated signaling pathways to be responsible for downregulation of α-defensins. Thus, attenuated expression of the anti-microbial peptides further highlights the role of immune evasion in *C. parvum* induced carcinogenesis. Moreover, identification of immune suppressive signaling molecules such as *ARG1, IDO1* and *SPP1*, characterized *C. parvum* to develop an immunosuppressive tumor microenvironment. In conclusion, based on the molecular data generated in the present study, systematic inflammation associated with chronic infection can be predicted to contribute to the *C. parvum*-induced immunosuppressive tumor microenvironment.

Results

Article 2

Persistent *Cryptosporidium parvum* infection leads to the development of tumor microenvironment in an experimental mouse model: Results of a microarray approach

Manasi Sawant[1], Sadia Benamrouz-Vanneste[1,2], Anthony Mouray[3,4], Peggy Bouquet [4], Nausicaa Gantois [1], Colette Creusy[5], Erika Duval[5], Adriana Mihalache[5], Pierre Gosset[5], Magali Chabé[1], David Hot[4], Eric Viscogliosi[1] and Gabriela Certad[1,6*]

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France

[2] Laboratoire Ecologie et Biodiversité, Unité Smart and Sustainable Cities, Université Catholique de Lille, F-59000 Lille, France.

[3] Plateforme d'Expérimentations et de Hautes Technologies Animales, Institut Pasteur de Lille, F-59000 Lille, France.

[4] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, US 41 - UMS 2014 - PLBS, F-59000 Lille, France

[5] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France

[6] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France.

[*] Correspondence : gabriela.certad@pasteur-lille.fr

Results

Abstract:

Cryptosporidium spp. are enteric protozoa parasites that infect a variety of vertebrate hosts. These parasites are capable of inducing life-threatening gastrointestinal disease in immunocompromised individuals. With the rising epidemiological evidence of the occurrence of *Cryptosporidium* infections in humans with digestive cancer, the tumorigenic potential of the parasite has been speculated. In this regard, *Cryptosporidium parvum* has been reported to induce digestive adenocarcinoma in a rodent model of chronic cryptosporidiosis. However, the processes by which the parasite could induce this carcinogenesis are still unknown. Therefore, the transcriptomes of *C. parvum* infected ileo-cecal regions of mice developing tumors were analyzed in the current study. For the first time, downregulation of the expression of α-defensin, an anti-microbial target of the parasite in response to *C. parvum* infection was observed in the transformed tissues. This phenomenon has been speculated to be the result of resistance of *C. parvum* to the host defense through the upregulated expression of interferon γ-stimulated genes. The inflammatory response generated as result of attenuated expression of anti-microbial peptides highlights the role of immune evasion in the *C. parvum*-induced tumorigenesis. The study has also succeeded in the characterization of the tumor microenvironment (TME) which is characterized by the presence of cancer associated fibroblasts, myeloid-derived suppressor cells, tumor-associated macrophages and extracellular matrix components. Identification of immune suppressor cells and accumulation of pro-inflammatory mediators speculates that chronic inflammation induced by persistent *C. parvum* infection assists in development of an immunosuppressive tumor microenvironment.

Keywords: Apicomplexa; *Cryptosporidium*; animal model; transcriptome; anti-microbial peptides; α-defensins; inflammation; tumor microenvironment; colon cancer

Results

Introduction

The apicomplexan parasite *Cryptosporidium* is recognized as one of the main waterborne agents causing diarrhoea worldwide. This ubiquitous intracellular parasite is responsible of self-limited diarrhoea in immunocompetent individuals but is capable of causing life-threatening disease in those who are immunocompromised [1]. Different cohort studies have reported that *Cryptosporidium* is one of the main pathogens responsible for severe diarrhea and mortality in children under 5 years old [2,3]. In addition, more than 200 outbreaks have been reported worldwide due to contaminated recreational or drinking water [4–6]. The low number of parasites required for an infection [7] coupled with the well-known resistance of *Cryptosporidium* oocysts to disinfection methods facilitates the waterborne transmission of cryptosporidiosis [8,9]. As a result, an ever-growing number of persons could be exposed to this parasite around the world. However, despite its prevalence and impact on public health, neither treatment nor vaccine against *Cryptosporidium,* are yet available [10].

Strikingly, *Cryptosporidium* has been identified with a significant higher prevalence among Lebanese [11], Chinese [12], and Polish [13] patients with colon cancer before undergoing any oncological treatment. The prevalence of *Cryptosporidium spp.* in colon cancer patients has been mainly restricted to *C. parvum* [11–13] and *C. hominis* [11]. Some additional reports showed that immunocompromised individuals suffering from HIV infection and cryptosporidiosis are at high risk of colorectal cancer (CRC) compared to HIV patients without cryptosporidiosis [14-15], indirectly suggesting that *Cryptosporidium* infection might somehow be associated with development of digestive cancer.

This causal link between *C. parvum* infection and cancer has been explored in an experimental model of cryptosporidiosis. In this severe combined immunodeficiency (SCID) rodent model, *C. parvum* infection was able to induce ileo-caecal adenocarcinoma [16–18]. The carcinogenic potential of this parasite was also confirmed in enteric explants [19]. The *C. parvum*-induced transformation did not exhibit classical canonical trademarks of colon cancer [18]. In contrast, alterations in cellular expression of APC and β-catenin were reported along with prominent basolateral and cytoplasmic localization of β-catenin potentially correlated with the involvement of a non-canonical Wnt pathway [18].

In parallel, inflammatory monocytes recruited at the sub-epithelial spaces were reported to assist *C. parvum* in reducing the transepithelial resistance via delocalization of E-cadherin and β-catenin from the adherens junctions of intestinal epithelial cells (IECs) [20]. Thus,

inflammatory response generated by the infection could potentially disrupt epithelial barrier function and eventually result in chronic inflammation which could promote tumorigenesis. In this respect, activation of pro-inflammatory signaling pathway such as nuclear factor-kappa B (NF-κβ) by *C. parvum* has been shown to inhibit apoptosis of infected epithelial cells [21].

Interestingly, a mouse model of inflammation inducing carcinogenesis with deletion of MCC (mutated in colorectal cancer) gene, demonstrated the absence of hyperactivation of β-catenin pathway [22] likewise to *C. parvum* associated cancer in which β-catenin was not found in the nucleus of the transformed cells [18]. This study emphasized an example of inflammation inducing DNA damage in the absence of external carcinogens. Hence, observation of *C. parvum* infection driven cancer without recording the DNA damage caused by canonical mutations [18] suggests that the parasite can be regarded as an external carcinogen, which might contribute to chronic inflammation during the infection, leading to tumor development.

Several microarray data are available wherein the gene profiles of *C. parvum* infected versus uninfected cells or tissue have been compared. These studies have identified cancer related pathways such as Hedgehog [23], Wnt [23] and p38/MAPK [24] to be altered by the parasite for its survival. Considering that these studies were carried out in already transformed cells, it was difficult to categorize these alterations as to play a significant role in *C. parvum* infection-induced cancer development. In the current transcriptomics study, we aimed to decipher novel molecules and signaling pathways which could be considered as potential specific markers of *C. parvum* infection induced digestive cancer.

Results

2. Materials and Methods

2.1. *Cryptosporidium* oocysts

C. parvum strain IOWA oocysts were purchased from Waterborne, Inc. (New Orleans, LA, USA) and stored in phosphate-buffered saline (PBS) supplemented with penicillin, streptomycin, gentamycin, amphotericin B and 0.001% Tween 20 at 4 °C until use. Absence of bacteria and fungi was assured by testing the oocyst suspensions on Plate Count Agar and Sabouraud plates for 1 week at 37 °C. The oocyst viability was assessed as described previously [19]. The oocyst suspension was first loaded on the FAST READ 102® slide (Biosigma, Cona, Italy) and observed under a Zeiss optical microscope (Zeiss, Oberkochen, Germany) to determine the number of intact and empty oocysts. The viability of oocysts was subsequently calculated by determining the ratio between the number of empty oocysts and the total number of intact and empty oocysts × 100.

2.2. Mouse Model of Cryptosporidiosis

Twenty-four seven-week-old CB17-SCID mice were obtained from a colony bred and regularly controlled for infections (including *Helicobacter* spp.) at the Pasteur Institute of Lille (France). Animals were maintained under aseptic conditions in an isolator during the whole experimentation, in a 12 h light-dark cycle with water and a standard diet (65% carbohydrate, 11% fat, and 24% protein; SAFE, Augy, France). Mice were administered with 4 mg/L of dexamethasone (Merck, Lyon, France) through drinking water. Dexamethasone treatment started 2 weeks prior to inoculation with the parasite and maintained during the entire experimentation. Dexamethasone-containing water was replaced three times a week. Infective doses of *C. parvum* (10^5 oocysts/mouse) were prepared and inoculated by intra-gastric feeding. Control animals were inoculated with PBS. Assessment of the clinical conditions of the mice was performed regularly to detect and then minimize suffering. Clinical signs that could constitute an endpoint included, but were not limited to, rapid or progressive weight loss, debilitating diarrhea, rough hair coat, hunched posture, lethargy or any condition interfering with daily activities (e.g., eating or drinking, ambulation or elimination). During the course of the experiment, 1 uninfected and 1 infected mice were found dead. The experiment was pursued with the remaining 22 mice which were distributed into 4 groups as follows: Group A (one uninfected mouse and two infected mice at 45 day post-infection (PI) for histological analysis), Group B (one uninfected mouse and one infected mouse at 93 day PI), Group C (four uninfected mice and five infected mice at 45 day PI for microarray analysis), Group D (three

uninfected mice and five infected mice at 93 day PI for microarray analysis). Mice were euthanized by carbon dioxide inhalation and samples from the ileo-cecal region were collected. At 45 day PI, entire ileo-cecal region was retrieved whereas at 93 day PI, polypoid masses visible macroscopically and measuring approximately 2.5 mm in diameter were harvested. Experiments were conducted in the animal facility at the Institute Pasteur of Lille (research accreditation number, D 59 350 009). Animal protocols were approved by the French regional ethical committee with the number APAFIS#9621.

2.3. Oocyst Shedding

The oocyst shedding was evaluated by collecting freshly excreted fecal pellets from each mouse at the time of euthanasia. Total genomic DNA was extracted from 200 mg of feces by using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The presence of parasite was detected by using the TaqMan real time PCR (qPCR) assay as previously described [25]. Briefly, the assay amplified a DNA fragment located in the 18S rRNA gene locus (GenBank accession no. EU675853, positions 33 to 211). The forward (5′-CATGGATAACCGTGGTAAT-3′) and reverse (5′-TACCCTACCGTCTAAACTG-3′) primers were designed to amplify a 178 bp fragment. A TaqMan probe homologous to a conserved region of the sequence (Pan-crypto probe; FAM-CTAGAGCTAATACATGCGAAAAAA-MGB-BHQ [FAM, 6-carboxy-fluorescein; MGB, minor-groove-binding ligand; BHQ, black hole quencher]) was designed to detect *Cryptosporidium*. The qPCR reactions were performed on a Rotor-Gene 6000 instrument (Corbett Research, Qiagen) with 50 ng of extracted DNA, 200 nM of each primer, 100 nM of the probe and 1X of Light Cycler 480 Probes Master. The gene amplification consisted of activation of the *Taq* DNA polymerase for 10 min at 94 °C, followed by 45 cycles of 94 °C for 10 s, 54 °C for 30 s and 72 °C for 10 s. Fluorescence signal was acquired at end of elongation step and the amplification data were analyzed using Rotor-gene Q Series software. The results were displayed using GraphPad Prism (version 9.1.0 (221), latest update on 2021/03/15)

2.4. Histological Examination

Ileo-cecal regions of mice from groups A and B were recovered then fixed in 4% buffered formalin. Formalin-fixed and paraffin-embedded specimens were sectioned at a thickened of 4 µm, stained with hematoxylin, eosin and saffron and examined microscopically for the detection of histological modifications of the host tissue. Pathological changes found in the mouse caecum were classified according to the Vienna classification of tumors of the digestive

system in humans [26] taking into account the nomenclature for histological assessment of intestinal tumors in rodent models [27] as previously described [28].

2.5. Agilent Microarray Analysis

The SurePrint G3 Mouse GE 8x60K Microarray and services (Agilent Technologies, Santa Clara, CA, USA) were used to process the samples and perform genome-wide analysis. Briefly, at specific time points, the individual mice from groups C and D were euthanized and the caecum tissue was harvested then placed in four volumes of RNAlater (Qiagen, Valencia, CA, USA) before storage at −80 °C. Total RNA was isolated from tissue using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNAse I (Sigma Life Sciences, St. Louis, MO, USA) according to manufacturer's protocol. RNA quality and quantity were determined using Agilent RNA6000 Nano kit by capillary electrophoresis and Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (100 ng) from each sample was labelled as described in One color microarray based gene expression analysis protocol (Agilent Technologies) using the Agilent Quick-Amp Labeling kit according to the manufacturer's instructions. After purification using a RNeasy Mini Kit (Qiagen), cRNA yield and incorporation efficiency (specific activity) into the cRNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, a total of 600 ng of cRNA was fragmented and hybridized overnight at 65 °C. After hybridization, slides were washed before being scanned on a SureScan Microarray Scanner (Agilent Technologies) and further processed using Feature Extraction v10.7.3.1 software. The resulting text files were uploaded into language R v4.0.3 and analyzed using the Linear Model for Microarray Data (LIMMA) package [29,30]. A 'within-array' normalization was performed using LOWESS (locally weighted linear regression) to correct for dye and spatial effects [31]. Moderate t-statistic with empirical Bayes shrinkage of the standard errors [32] was then used to determine significantly modulated genes. Statistics were corrected for multiple testing using a false-discovery rate approach.

2.6. Data Analysis

To visualize and explore the molecular interaction networks of the differentially expressed genes, the subsequent data were uploaded into the Ingenuity Pathways Analysis (IPA) software (QIAGEN CLC Genomics Workbench 20.0; version 68752261; latest update on 6 September 2021) (https://digitalinsights.qiagen.com/) (Qiagen, Redwood City, CA, USA) to organize the differentially expressed genes into networks based on the Ingenuity Knowledge Database

(IKB), an extensive database of functional direct and indirect interactions between genes from peer-reviewed publications. Particularly, the present study applied IPA system to uncover the signaling pathways, interactions and functional roles associated with differentially expressed genes in *C. parvum* infected caecum tissues in comparison with controls. IPA uses a network generation algorithm to segment the network map between molecules into multiple networks and assign scores for each network. The score is generated based on hypergeometric distribution, where the negative logarithm of the significance level is obtained by Fisher's exact test at the right tail [33]. For upstream regulators, disease and function, the −log (*p*-value) > 4 was taken as threshold, the z-score > 2 was defined as the threshold of significant activation, whilst z-score < −2 was defined as the threshold of significant inhibition. For upstream regulators, the *p*-value of overlap < 0.05 was also set as the threshold. For regulator effects, consistency scores were calculated, with a high consistency score indicating accurate results for the regulatory effects analysis. The algorithm used for calculating the z-scores and *p*-values of overlap has been described previously [34]. The graphs were produced used GraphPad Prism (version 9.1.0 (221), latest update on 2021/03/15)

2.7. Validation by Quantitative Reverse Transcription PCR

Quantitative Reverse Transcription PCR (RT-qPCR) was used to validate microarray results of four upregulated and two downregulated genes. cDNA was synthesized from 1 µg of total RNA extracted from the caecum tissue using oligo-$(dT)_{20}$ primer and Superscript III reverse transcriptase (RT) in a 20 µL reaction (Invitrogen) according to the manufacturer's instruction. RT-qPCR was performed with a Corbett Research RG-6000 Real time PCR machine (Qiagen). Primers used are listed in supplementary Table S1and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. Each RT-qPCR reaction (20 µL) contained 7.5 µL Brilliant III Ultra-Fast qPCR mastermix (Agilent Technologies), 400 nM of each forward and reverse primers and 50 ng of template cDNA. PCR amplification consisted of activation of the *Taq* DNA polymerase for 3 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, 57 °C for 20 s and 72 °C for 20 s. Fluorescence signal was acquired at the end of elongation step and the amplification data were analyzed using Rotor-gene Q series software. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of target genes in infected mice with the constitutively expressed endogenous control and the ΔC_t value of the uninfected mice was used as the calibrator [35]. The results were displayed using GraphPad Prism (version 9.1.0 (221), latest update on 2021/03/15).

3. Results

3.1. *C. parvum* Induces Chronic Infection and Neoplasia in a Mouse Model

Using the well-documented model of intestinal cryptosporidiosis (SCID mice treated with dexamethasone) through the oral administration of the oocysts, infection of *C. parvum* was detected by quantification of the oocyst shedding (Figure 1A). Upon histological examination of the ileo-cecal region (Figure 1B), well-differentiated adenocarcinoma processes invading the submucosae were observed in two out of seven mice that were euthanized at day 45 PI. Glands with necrotic content and a large number of polymorphic inflammatory elements were also observed. Mice euthanized at day 93 PI presented polypoid, sessile, masses, measuring approximately 2.5 mm in diameter in the ileocecal region. At histological examination, we observed an invasive well-differentiated adenocarcinomas that progressed into the lamina propria (intramucosal carcinoma, category 4.4 Vienna classification) in one out of six and in the other five a well-differentiated adenocarcinoma penetrating the inner layer of the muscularis (category 5 Vienna classification) with a desmoplastic response around the glands, which is a typical feature of invasive carcinoma in rodents. Necrosis of the glands and numerous polymorphic inflammatory elements in the chorion were also described. Presence of numerous parasites at different developmental stages in the lumen of the glands were also reported at days 45 and 93 PI.

Results

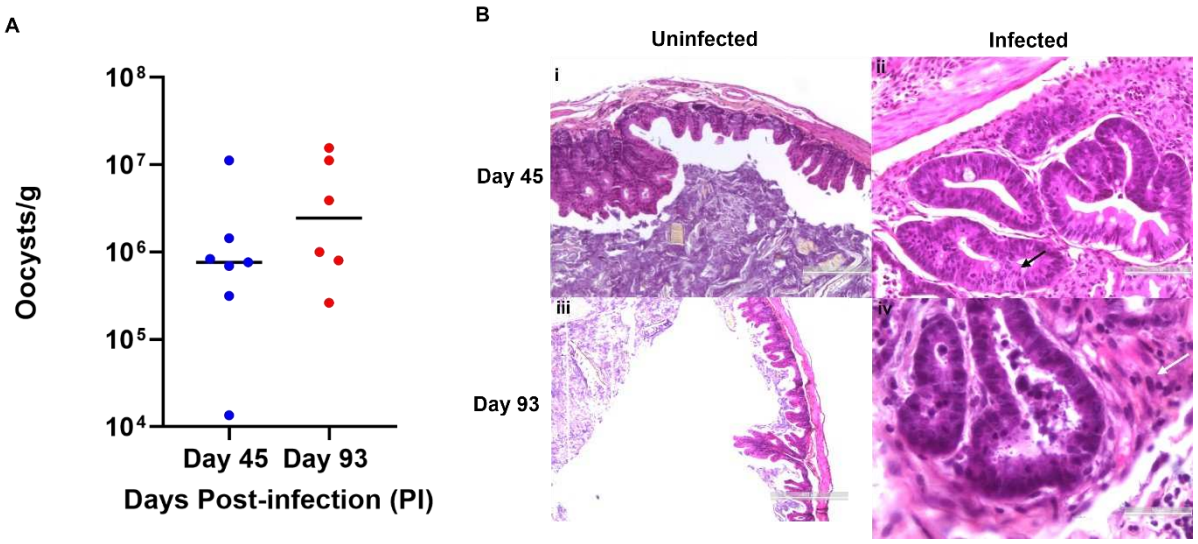


Figure 1. Experimental SCID mouse model of chronic cryptosporidiosis and neoplasia. (**A**) Pattern of oocyst shedding (oocysts/g of faeces) in mice at days 45 (blue dots) and 93 PI (red dots) wherein each dot represents individual mouse. The line in each pattern corresponds to the geometric mean of oocyst shedding per group. (**B**) Histological examination of *C. parvum* uninfected (i–iii) and infected (ii–iv) ileo-caecal regions at days 45 and 93 PI. Presence of significant cytonuclear atypia and appearance of intra-mucosal adenocarcinoma are observed at day 45 PI (black arrow). Desmoplastic reaction is also reported around the invasive neoplastic glands (white arrow) at day 93 PI.

3.2. Chronic Cryptosporidiosis Induced Neoplasia Results in Global Gene Profile Alterations

The gene expression profiles were significantly altered with 43 and 931 genes reported to be differentially regulated at day 45 PI and day 93 PI, respectively (LogFC2.0; adj *p*-value < 0.05) as shown in Table 1. The entire lists of regulated genes are provided as Supplementary Tables S2 and S3.

Fold changes of infected mice at day 45 PI and day 93 PI were compared with those of control uninfected animals of matching genotype with significant adj *p*-value (<0.05).

Table 1. Altered expression of highly regulated genes at days 45 and 93 PI. Fold changes of infected mice at day 45 PI and day 93 PI were compared with those of control uninfected animals of matching genotype with significant adj p-value (<0.05).

Gene Symbols and Names	Direction of regulation	LogFC day 45	LogFC day 93
Iigp1; interferon inducible GTPase 1	Up	6.78	5.74
Defa4; defensin, alpha, 4	Up (day 45 PI) Down (day 93 PI)	6.37	-6.07
H2-DMb1;histocompatibility 2, class II, locus Mb1	Up	6.35	3.20
Tgtp2; T cell specific GTPase 2	Up	6.31	5.13
Cd74; CD74 antigen	Up	6.23	2.99
H2-Ab1; histocompatibility 2, class II antigen A, β	Up	6.21	2.91
H2-Eb1; histocompatibility 2, class II antigen E, β	Up	5.89	3.27
Ciita; class II transactivator	Up	5.63	2.73
H2-Aa; histocompatibility 2, class II antigen A, α	Up	5.31	2.56
UBD; ubiquitin D	Up	5.15	4.25
Ido1; indoleamine 2,3-dioxygenase 1	Up	5.07	4.41
Cxcl10; chemokine (C-X-C motif) ligand 10	Up	4.56	4.44
Gbp11; guanylate binding protein 11	Up	4.39	3.11
Cxcl9; chemokine (C-X-C motif) ligand 9	Up	4.38	3.68
Gbp4; guanylate binding protein 4	Up	4.14	2.81
Gbp2b; guanylate binding protein 2b	Up	3.90	2.93
Gbp6; guanylate binding protein 6	Up	3.90	3.20
Il18bp; interleukin 18 binding protein	Up	3.68	3.21
Gbp8; guanylate-binding protein 8	Up	3.60	2.06
Ly6a; lymphocyte antigen 6 complex, locus A	Up	3.35	5.52
Igtp; interferon gamma induced GTPase	Up	3.37	2.90
Gbp2; guanylate binding protein 2	Up	3.32	2.92
Spp1; secreted phosphoprotein 1	Up	3.04	5.07
Ly6e; lymphocyte antigen 6 complex, locus E	Up	2.13	5.25
S100a9; calgranulin B	Up	N/A[a]	9.14
S100a8; calgranulin A	Up	N/A[a]	8.42
Mmp10; matrix metallopeptidase 10	Up	N/A[a]	6.76
Il1b; interleukin 1 beta	Up	N/A[a]	6.74
Defa2; defensin, alpha, 2	Down	N/A[a]	-6.02
Il1rl1; interleukin 1 receptor-like 1	Up	N/A[a]	5.84
Ifi44l; interferon-induced protein 44 like	Up	N/A[a]	5.77
Arg1 ; arginase	Up	N/A[a]	5.34
Defa3; defensin, alpha, 3	Down	N/A[a]	-5.76
Mmp8; matrix metallopeptidase 8	Up	N/A[a]	5.74
Il11; interleukin 11	Up	N/A[a]	5.74
Mmp13; matrix metallopeptidase 13	Up	N/A[a]	5.49
Defa1; defensin, alpha 1	Down	N/A[a]	-5.76

Ifi202b; interferon activated gene 202B	Up	N/A[a]	5.27
Cxcl1; chemokine (C-X-C motif) ligand 1	Up	N/A[a]	5.27
Ccl3; chemokine (C-C motif) ligand 3	Up	N/A[a]	5.23
Ccl2; chemokine (C-C motif) ligand 2	Up	N/A[a]	5.19
Slc37a2; solute carrier family 37 (glycerol-3-phosphate transporter), member 2	Down	N/A[a]	-4.50
Col1a1; collagen, type I, alpha 1	Up	N/A[a]	4.87
Il1r2; interleukin 1 receptor, type II	Up	N/A[a]	4.95
Adam8; a dis integrin and metallopeptidase domain 8	Up	N/A[a]	4.89
Cxcl5; chemokine (C-X-C motif) ligand 5	Up	N/A[a]	4.87
Sult1c2; sulfotransferase family, cytosolic, 1C, member 2	Down	N/A[a]	-4.68
Ifit2; interferon-induced protein with tetratricopeptide repeats 2	Up	N/A[a]	4.63
Mmp3; matrix metallopeptidase 3	Up	N/A[a]	4.58
Cyp2c40; cytochrome P450, family 2, subfamily c, polypeptide 40	Down	N/A[a]	-4.33
Cxcl2 ; chemokine (C-X-C motif) ligand 2	Up	N/A[a]	4.14
Col1a2; collagen, type I, alpha 2	Up	N/A[a]	4.14
Ptgs2 ; prostaglandin-endoperoxide synthase 2	Up	N/A[a]	4.04

The experimental datasets from each time point were subjected to core analysis using IPA software. Analysis was performed to characterize the global functions associated with the altered gene profiles. As a result, 37 genes at day 45 PI and 900 genes at day 93 PI were mapped using the software. The software predicted 35 genes to be "analysis-ready molecules" at day 45 PI wherein 33 of them were predicted as upregulated and the two remaining ones as downregulated. At day 93 PI, 758 "analysis-ready molecules" were predicted wherein 566 and 192 genes were predicted to be upregulated and downregulated, respectively.

As a part of core analysis, the experimental datasets were also subjected to different features of the software which included "upstream regulators", "diseases and functions" and "regulatory effects" analysis. The upstream regulators are the predicted molecules involved in the regulation of gene expression changes within the datasets. By applying the threshold of overlap p-value < 0.05, a total of 41 and 306 upstream regulators were identified at day 45 PI and day 93 PI, respectively. The overlap p-value measures whether a statistically significant overlap is pointed out between the dataset genes and the genes that are regulated by the transcriptional regulator. Among them, 29 (activated) had activation z-score > 2 and 12 (inhibited) had activation z-score < −2 at day 45 PI whereas about 140 upstream regulators were predicted to be activated and about 166 inhibited at day 93 PI. The top three upstream regulators identified at both time points were Lipopolysaccharide (*LPS*), Interferon-γ (*IFNγ*) and Tumor necrosis factor (*TNF*) (Figure 2A). *IFNγ* was observed to be the most powerful activator at day 45 PI (Z-score = 3.954, overlap p-value = 2.97×10^{-15}, 16 target molecules) whereas *LPS* was identified as the most powerful activator at day 93 PI (Z-score = 11.165, overlap p-value = 3.23

$\times 10^{-93}$, 277 target molecules). Further, by applying the −log (*p*-value) > 4 threshold, the top diseases and cellular functions associated with the molecules in the datasets were determined. As shown in Figure 2B, the top four "Diseases and Disorders" observed to be common at both time points were as follows: "Gastrointestinal Disease" [day 45 PI (*p*-value = 8.06×10^{-16}), day 93 PI (*p*-value = 1.90×10^{-54})], "Immunological Disease" [day 45 PI (*p*-value = 8.04×10^{-16}), day 93 PI (*p*-value = 4.11×10^{-68})], "Inflammatory Response" [day 45 PI (*p*-value = 1.61×10^{-21}), day 93 PI (*p*-value = 8.61×10^{-64})] followed by "Organismal Injury and Abnormalities" [day 45 PI (*p*-value = 1.06×10^{-23}), day 93 PI (*p*-value = 8.61×10^{-64})]. Out of the top four, "Organismal Injury and Abnormalities" was reported to have highest number of molecules to be involved from each dataset (day 45 PI = 30 molecules and day 93 PI = 582 molecules). Moreover, regulatory effects analysis algorithm was used to connect the upstream regulators, dataset molecules and downstream disease and functions to generate a hypothesis of how a particular function is regulated in the dataset. The regulator effects are determined in terms of a consistency score which is a measure of how casually consistent and densely connected a regulatory network is. The upstream regulators and diseases and functions included in the analysis were of −log (*p*-value) > 4 and z-score > 2. As a result of this analysis, at day 45 PI the highest ranked regulators were observed to be *CD28* and cytochrome p450 oxidoreductase (*POR*) with a consistency score of 2.121 which may be involved in the inhibition of cellular function of mammalian infection mainly through mediating their targets which encode regulatory proteins such as *UBD* (ubiquitin D), serine proteases such as Granzyme A (*GZMA*), IFNγ-induced GTPases such as Guanylate binding protein 2 (*GBP2*) and molecules associated with major histocompatibility complex (MHC) class II such as *CD74, HLA-DQB1, HLA-DMA* and *CIITA* (Figure 2C). At day 93 PI, Tumor necrosis factor ligand superfamily member 12 (*TNFSF12*), also known as TNF-related weak inducer of apoptosis (TWEAK), was observed with a highest consistency score of 4.243 to be involved in the activation of cellular function of inflammatory response via mediating the targets such as matrix metalloproteinases (*MMP*, *ADAM8*), chemokines and cytokines (*CCL7*, *CXCL10, CCL11*, *CCL3L3*, *CXCL2*, *CXCL6*, *CCL2*, *CXCL3*, *S100A8*, *S100A9*, *IL1β*, *IL6* and *TNF*) and immunoglobulins (*ICAM1* and *VCAM1*) (Figure 2D).

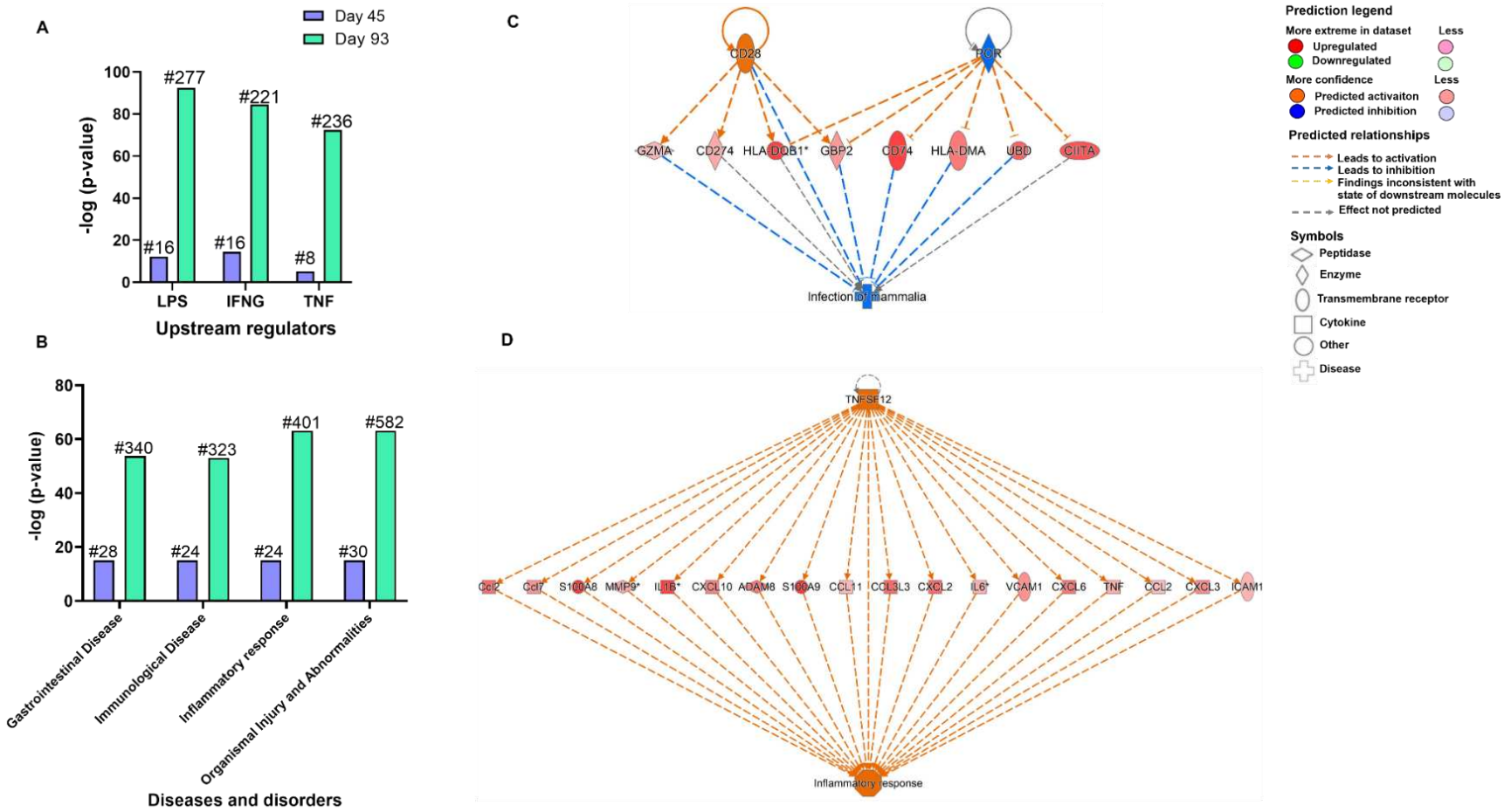


Figure 2. IPA analysis of transcriptomes of *C. parvum* infected caecum tissue when compared to uninfected tissue at days 45 and 93 PI. (**A**) Top three common upstream regulators observed at days 45 and 93 PI are represented in terms of −log (*p*-value) on y-axis. (**B**) Top four common diseases and disorders observed at days 45 and 93 PI represented in terms of −log (*p*-value) on y-axis (# = no. of differentially expressed genes). (**C**) Molecular network diagram representing the top regulatory pathways predicted to be involved in the function mammalian infection at day 45 PI with a consistency score of 2.121. (*) Indicates that multiple identifiers are present in the dataset file which map to a single gene in the global molecular network. (**D**) Molecular network diagram representing the top regulatory pathways predicted to be involved in inflammatory response at day 93 PI with a consistency score of 4.213.

Results

3.3. Regulation of Anti-Microbial Peptides Like α-Defensins during the Course of C. parvum Infection

As previously described, mammalian infection was observed to be a highly downregulated function at day 45 PI (z score = −2.017). Parasitic infection was just following the mammalian infection function with a z-score of −1.969. Out of the 13 molecules identified to be involved in mammalian infection, eight of them were in common with the parasitic infection function (Figure 3A). Interestingly, the expression levels of some highly regulated genes predicted to inhibit parasitic infection which include antigen representing MHC class II molecules (*H2-AB1*, *H2-AA*, *CIITA* and *H2-EB1*), IFNγ induced GTPases (*IIGP1*, *IGTP*, *GBP2*, *GBP8*, *GBP11*, *GBP6* and *GBP4)* and chemokines *(CXCL10* and *CXCL9)* does not differ between day 45 PI and day 93 PI (Figure 3B). At day 93 PI, the experimental dataset revealed downregulation of the chemokine *CCL20*, known to exert anti-microbial activity against *C. parvum* (Figure 3B). The analysis also identified α-defensins 4 (*DEFA4*), an anti-microbial peptide, to be highly upregulated at day 45 PI (Log FC = 6.37) compared to day 93 PI (Log FC = −6.07) wherein it was highly downregulated (Figure 3B). Other isoforms of α-defensins, *DEFA1*, *DEFA2 and DEFA3* (α-defensins 1, 2 and 3, respectively) were also observed to be highly downregulated.

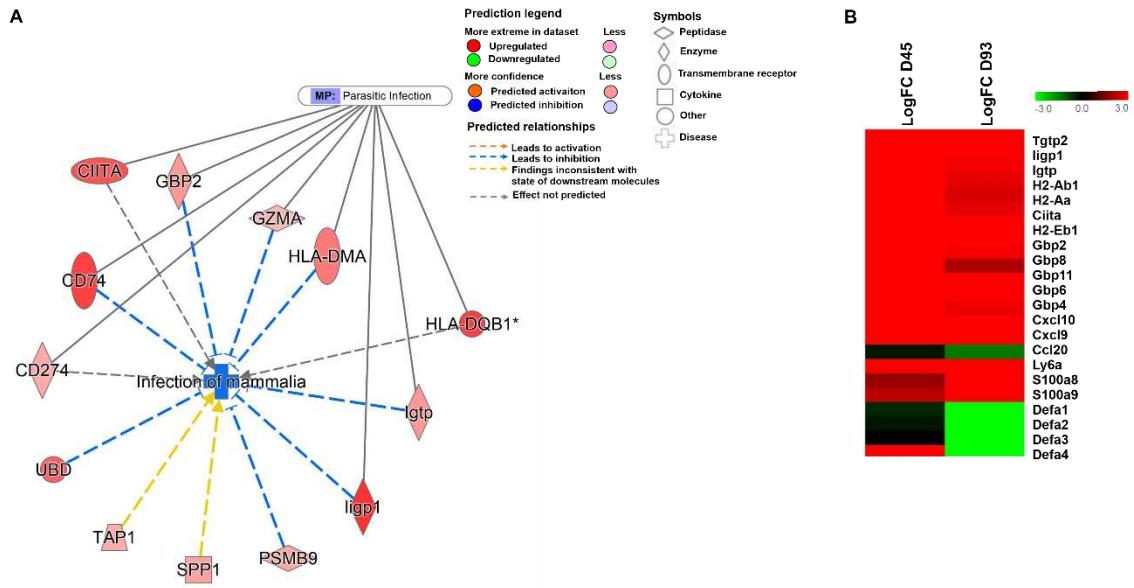


Figure 3. Analysis of molecules regulated during *C. parvum* infection. (**A**) Molecular network representing genes involved in mammalian infection overlaid with the function of parasitic infection. (*) Indicates that multiple identifiers are present in the dataset file which map to a single gene in the global molecular network. (**B**) Heatmap of genes significantly upregulated or downregulated in infected caecum tissue compared to uninfected tissue at days 45 and 93 PI.

Results

Using the tool "My Pathways" available in IPA, customized pathways were created for target molecules of interest including *DEFA4, DEFA1, CIITA* and *IIGP1*. Interferon-inducible GTPase 1 (*IIGP1*) and class II, major histocompatibility complex, transactivator (*CIITA*) have been selected to showcase the network of genes known to be involved in inhibition of parasitic infection. *Cryptosporidium* is speculated to circumvent this action. *DEFA1* and *DEFA4* have been selected to identify the network of genes regulating their expression considering α-defensins as potential novel targets of *C. parvum* to persist the infection. The network for each target molecule was built on the molecules and/or relationships available in the database exclusively from epithelial cells, small intestine and large intestine. Further, the developed pathways were overlaid with experimental datasets (Log FC2 adj *p*-value 0.05) at day 45 PI and day 93 PI to measure the expression level of the molecules. As a result of this analysis, *DEFA4* expression was observed to be indirectly regulated by intestinal epithelial insulin receptors (*INSR*). At day 45 PI, *INSR* is predicted to be inhibited which results in upregulation of *DEFA4* in intestinal epithelial cells (Figure 4A). However, at day 93 PI, we observe an increase in the expression of *IGF1* (insulin growth factor 1) which is predicted to activate *INSR*, resulting in downregulation of *DEFA4* expression. Predicted activation of *INSR* is also supported by downregulation of other genes from the experimental dataset observed in the network such as hydroxyprostaglandin dehydrogenase (*HPGD*), pyruvate dehydrogenase (*PDK4*), *PCK1* (phosphoenolpyruvate carboxykinase 1), aldehyde dehydrogenase family member A 1 (*ALDH1A1*) and aldoketoreductase family 1 member B7 (*Akr1b7*) (Figure 4B). The second hypothetical network predicted *DEFA1* expression to be regulated by TNF receptor associated factor 2 (*TRAF2*). At day 45 PI, *DEFA1* was predicted to be downregulated with very low confidence score due to predicted inhibition of *TRAF2* (Figure 4A). However, at day 93 PI, with the increase in the expression levels of cytokines such as Interleukin 1β (*IL1β*) and *TNF*, *DEFA1* was predicted to be downregulated by *TRAF2* with a high confidence score (Figure 4B). Apart from the cytokines, a transcriptional factor such as Signal Transducer and Activator of Transcription 1 (*STAT1*) [z-score = 3.343] was also predicted to inhibit *TRAF2* (Figure 4). In IECs, *STAT1* expression is dependent upon the transcription factor retinoic acid receptor β (*RARβ*) [z-score = 2.363]. Moreover, upregulation of several immune response molecules such as *IIGP1, IGTP, GBP2* and *IRGM1* in the IECs was predicted to be dependent upon *RARβ* (Figure 3). In addition, *STAT,* is also regulated by one of the highly inhibited upstream regulators called *POR* [z-score = −2.813]. Downregulation of POR enzymes has been responsible for enhancing the expression of *STAT1* which is an activator of the class II transactivator for MHC II gene expression IECs (Figure 4). Thus, *POR* is indirectly responsible

for regulation of expression of *CIITA* along with the molecules of MHC II group such as *CD74*, *HLADQA1*, *HLADMA*, *HLADQB1* and *HLADRB5* previously identified as genes responsible for inhibition of parasite infection.

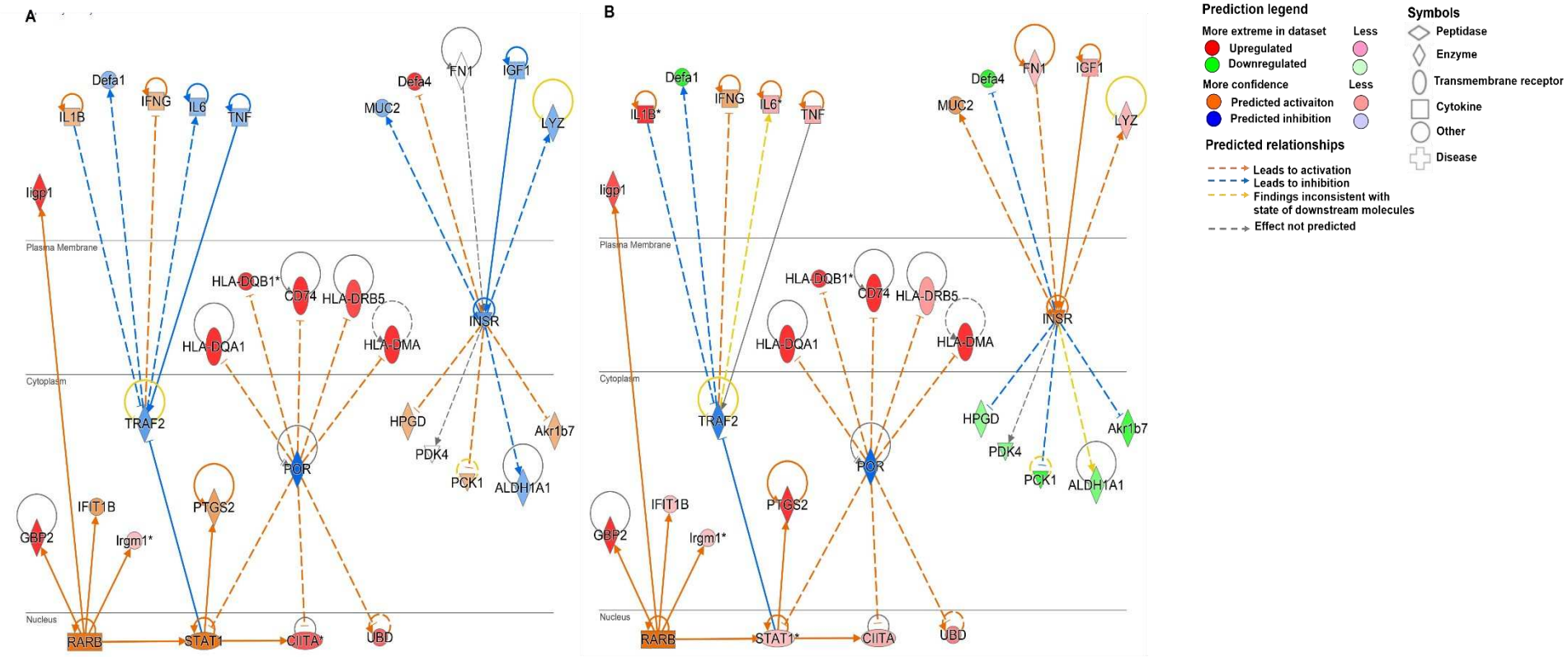


Figure 4. Gene network based analysis of role of α-defensins in *C. parvum* infection regulated by *INSR* and *TRAF2*. Overlay of molecular network generated with experimental datasets (LogFC2 adj *p*-value 0.05) from (**A**) day 45 PI and (**B**) day 93 PI. (*) Indicates that multiple identifiers are present in the dataset file which map to a single gene in the global molecular network.

Results

3.4. Chronic Cryptosporidiosis Induces Tumor Microenvironment

Experimental datasets from days 45 and 93 PI were subjected to comparative IPA analysis to identify enriched "Canonical Pathways" associated with cancer by applying the -log(*p*-value) > 2 threshold. As a result, cancer related canonical pathways were majorly identified in the dataset at day 93 PI compared to day 45 PI (Figure 5A). "*PD-L1* Cancer Immunotherapy Pathway" (*p*-value = 3.02×10^{-6}) was the only pathway identified within the dataset at day 45 PI. However, this pathway was predicted to be inhibited with an activation z-score of −2.0. Programmed cell death ligand-1 (*PD-L1*), also known as *CD274*, is an immune checkpoint molecule which is expressed by tumor cells or infiltrating myeloid cells to induce apoptosis of T cells and drive immune suppression which is highly expressed at day 45 PI (Log FC = 2.78).

The top ranked "Tumor Microenvironment Pathway" (*p*-value = 6.47×10^{-10}) was predicted to be activated with an activation z-score of 5.099 at day 93 PI. This pathway had an overlap score of 15.6% with 29/173 molecules found within the dataset. A heatmap was generated to identify the regulation of expression level of these overlapping genes. All the genes identified to activate the tumor microenvironment pathway were highly upregulated at day 93 PI except secreted phosphoprotein 1 (*SPP1*), Indoleamine 2,3-dioxygenase 1 (*IDO1*), *UBD* and *CD274,* which were also highly upregulated at day 45 PI (Figure 5B). The other major pathways identified in terms of regulated gene numbers were HOX antisense intergenic RNA) regulatory pathway (HOTAIR) and *HLA-F adjacent transcript 10*, also known as UBD) cancer signaling pathway (FAT10). The identification of other genes from the datasets that are associated with canonical pathways such as "Glioma Invasiveness Signaling Pathway" and "Role of Tissue Factor in Cancer" highlighted the invasive nature of the tumor development.

This result was further confirmed by the identification of "Organismal Injury and Abnormalities" as one of the highly regulated disease/disorder (Figure 2B). After applying a z-score > 2 as threshold, it was observed that most of the cellular functions that define this disease/disorder are associated with cancer. The heatmap generated for "Organismal Injury and Abnormalities", out of the 17 activated cellular functions with z-score > 2, 10 functions were associated with growth, metastasis, invasion and neoplasia of tumor. These 17 cellular functions are represented in terms of -log *p* values in Figure 5C. Invasive tumor (*p*-value = 9.37×10^{-54}) had the highest activation z-score of 4.013 with a total of 169 molecules involved in the dataset (Figure 5C).

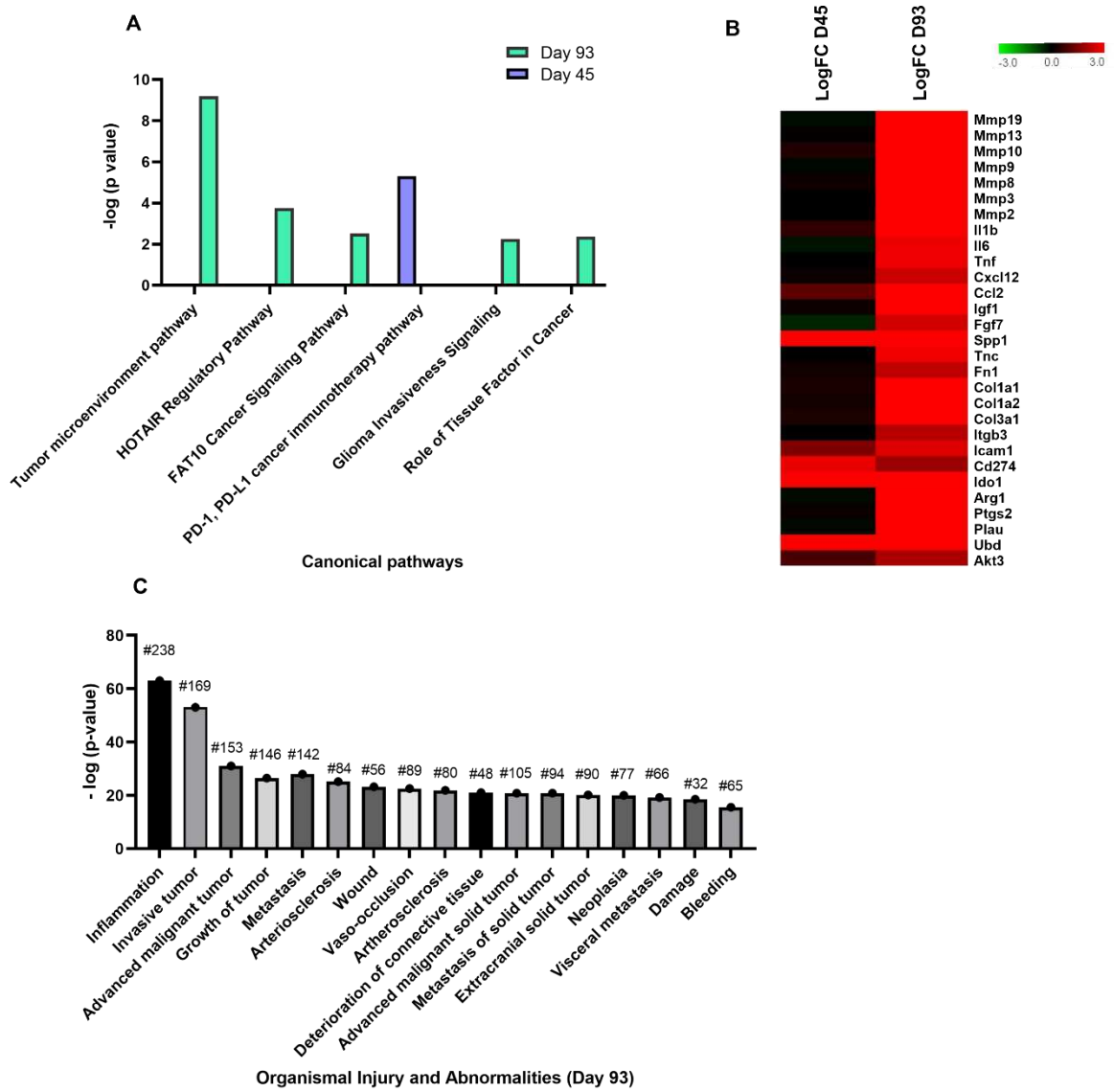


Figure 5. IPA analysis of tumor microenvironment induced by *C. parvum* infection. (**A**) Cancer related canonical pathways observed at days 45 and 93 PI are represented in terms of -log (*p*-value) on y-axis. (**B**) Heatmap of genes identified in tumor microenvironment canonical pathway at days 45 and 93 PI. (**C**) Cancer-related functions associated with "Disease/Disorder—Organismal Injury and Abnormalities" are represented in terms of −log (*p*-value) on y-axis. # = no. of differentially expressed genes.

Results

Using the tool "My Pathways", customized pathways were created to target molecules of interest (*TNF* and *STAT1*). The network for each target molecule was built on the molecules and/or relationships available in the databases exclusively from epithelial cells or cancerous epithelial cell lines. Further, the developed pathways were overlayed with experimental datasets (LogFC2 adj *p*-value 0.05) obtained at day 93 PI to detect the expression levels of the predicted molecules. As shown in Figure 6A, *TNF-α* expression was observed to upregulate expression of several cancer-associated genes in the epithelial cells which include extracellular matrix (ECM) remodeling genes such as *MMP9*, *PLAU*, *FN1* and *ICAM1*, chemokines such as *CCL2*, *CXCL2*, *CXCL3* and *CXCL10* and regulatory genes such as *UBD* and *PTGS2*. In IECs, expression of *STAT1* gene was also predicted to be upregulated by inflammatory cytokines *IL1β* and *IFNγ* and growth factor *FGF7* identified as key molecules involved in tumor microenvironment. Moreover, activation of *STAT1* has been predicted to also upregulate expression of cancer associated genes such as *IDO1* and *PTGS2* (Figure 6A).

Figure 6B illustrates a more detailed gene network predicted to be involved in the tumor microenvironment induced by *C. parvum* infection. Upregulated expression of cytokines and chemokines indicated functionality of tumor-associated macrophages within the tumor microenvironment. These cytokines have been identified to be directly or indirectly responsible for cancer progression through remodeling of ECM by upregulating the expression of molecules such as *MMP2*, *MMP3*, *MMP9, MMP10, MMP13*, *PLAU*, *ICAM1* and *VCAM1*. Upregulated expression of *SPP1* has been identified as a candidate to trigger the release of these MMPs to induce metastasis of cancer cells via *NF-κβ*. *NF-κβ* has also been observed as central regulatory molecule as it is predicted to be activated by most of the pro-inflammatory cytokines such as *TNF-α*, *IFNγ* and *IL-β*. Its activation results in the upregulated expression of enzyme involved in synthesis of prostaglandins (*PTGS2*), *UBD*, chemokine (*CXCL3*), serine proteases (*PLAU*) and metalloproteinases (*MMP10* and *MMP13*).

Activated expression of *UBD* has been predicted to downregulate the p53 tumor-suppressor gene. Along with *NF-κβ*, *STAT1* is another transcription factor observed to be central to *IFNγ*, *TNF-α* and *IL-1β* mediated signaling. The network identified immunosuppressive cell signaling molecules such as *IDO1* to be regulated by *STAT1.* Anti-microbial peptides such as *DEFA1* and *DEFA4* were predicted to be downregulated by *STAT1* signaling whereas *DEFA3* appeared to be downregulated due to inhibition of *IKBKG* (inhibitor of nuclear factor kappa B kinase regulatory subunit gamma) caused by overexpression of *IL1β*. By inducing upregulation of *IFNγ* regulated molecules such as *GBP5*, *STAT1* expression can be considered to play a role in

predicted activation of *IFNγ* along with other immune response molecules associated with parasitic infection such as *IIGP1*, *GBP2*, *GBP5*, *IRGM1* and *IFI16.* Activation of *IFNγ* can also be attributed to the detection of upregulated gene expression of Lymphocyte antigen 6 complex (*LY6C*), an antigen indicating the presence of inflammatory monocytes. Moreover, *IFNγ* along with *IL1β* was also predicted to negatively downregulate expression of nuclear receptor genes such as Vitamin D receptor (*VDR*) and Peroxisome Proliferator Activated Receptor Alpha (*PPARA*). These nuclear receptors especially *PPARA* in turn have been identified to negatively regulate the expression of tumor suppressor such as 3-Hydroxy-3-Methylglutaryl-CoA Synthase 2 (*HMGCS2*). The tumor microenvironment network also identified significant downregulation of genes associated with metabolism of hormones, drugs and xenobiotic compounds such as Cytochrome P450 Family 2 Subfamily C Member 18 (*CYP2C18*), *SLC10A2*, Solute Carrier Family 10, 27 Member 2 (*SLC27A2*), and Sulfotransferase Family 1C Member 2 (*SULT1C2*) which in turn is expected to inhibit *IL-10RA* (interleukin-10 receptor) expression. *IL-10*, an anti-inflammatory receptor is also predicted to be inhibited by immunosuppressor *SPP1*. Arginase1 (*ARG1*) is another immunosuppressive molecule identified in the network predicted to be regulated by *TGF-β* along with serine protease (*SERPINE1*) and metalloproteinase 13 (*MMP13*).

Results

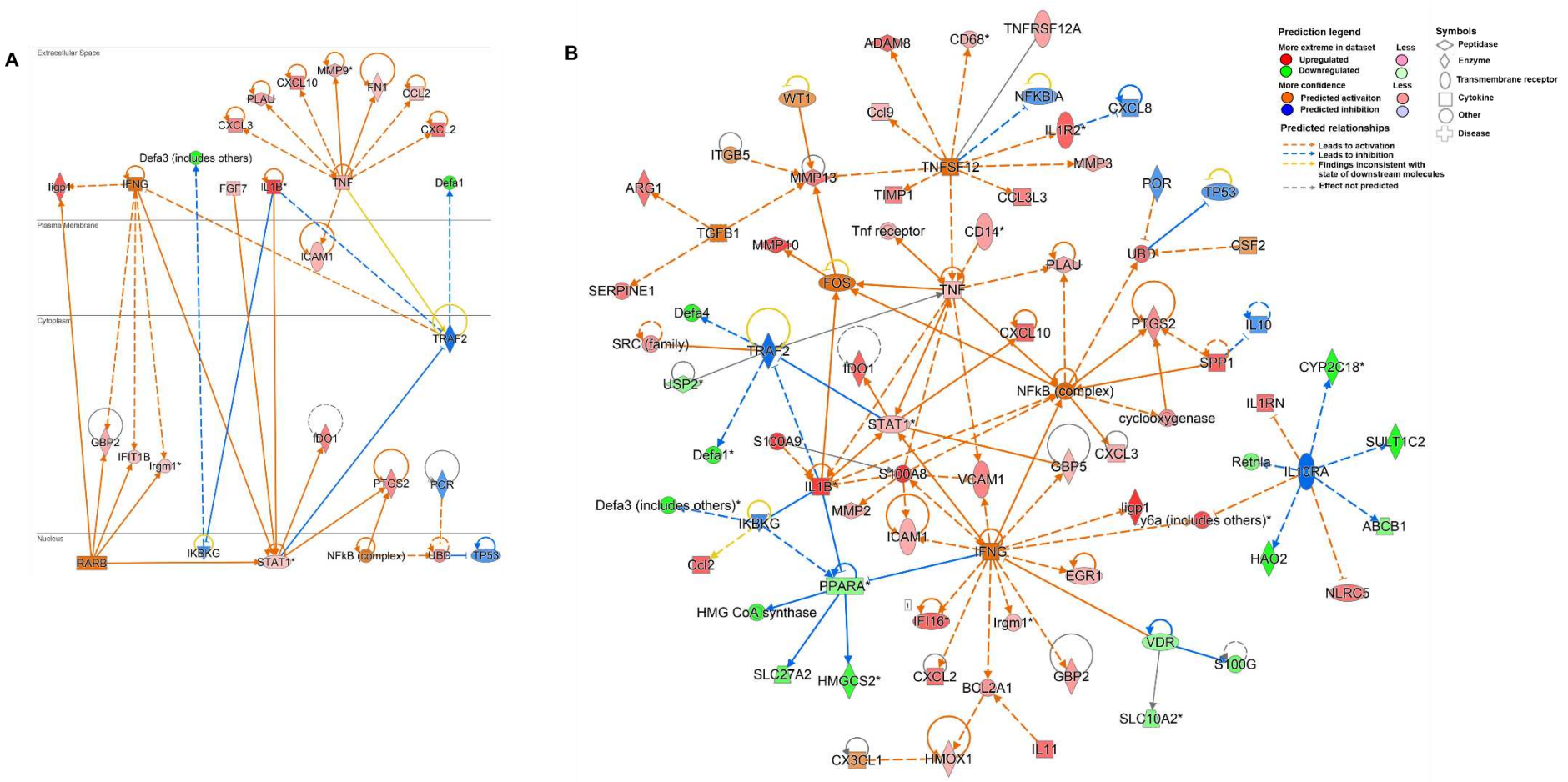


Figure 6. (**A**) Gene network based analysis of tumor associated genes in intestinal epithelial cells at day 93. (**B**) Customized gene network predicted within the tumor microenvironment induced by *C. parvum* infection. Overlay of molecular network is generated with experimental datasets from day 93 PI. (*) Indicates that multiple identifiers are present in the dataset file which map to a single gene in the global molecular network.

3.5. Validation of Target Genes by RT-qPCR

In total, six genes of interest were selected for RT-qPCR validation of microarray results at day 93 PI. These genes were chosen based on individual function speculated for them during *C. parvum* infection induced tumor development. *IIGP1* was chosen to validate the microarray results concerning the host immune defense against parasite infection, *IDO1*, *SPP-1* and *UBD* for the progression of tumor microenvironment, and *DEFA1* and *DEFA3* for the anti-microbial response speculated to be suppressed by *C. parvum* in order to propagate cancer. RT-qPCR analysis detected an average of 6.93, 7.83, 5.5 and 4.6 relative fold increases in the respective expression of *IIGP1*, *IDO1*, *SPP1* and *UBD* after normalization to the endogenous expression of *GAPDH* in the *C. parvum* infected caecum tissue. *DEFA1* and *DEFA3* expressions were observed to be decreased by a fold change of 0.5 (Figure 7).

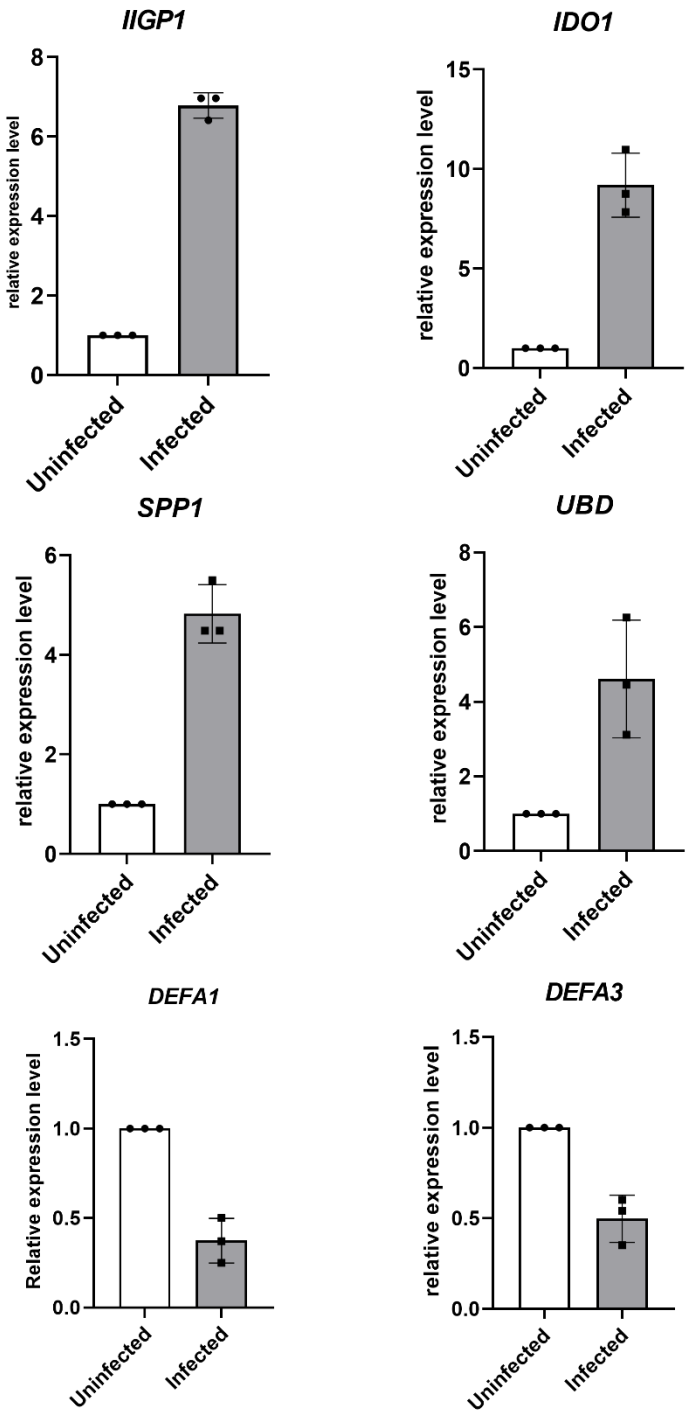


Figure 7. Validation of microarray data by RT-qPCR on a panel of seven targeted genes at day 93 PI. The expression levels were analyzed in triplicate and normalized to *GAPDH*. Each black circle and each black square indicates an individual uninfected and infected mouse respectively. The error bars indicate the mean with SD.

4. Discussion

In the current study, a transcriptomic approach was conducted to explore the signaling pathways potentially involved in the development of neoplasia induced by *C. parvum*. Global upregulation of genes associated with the innate immune response was observed despite the administration to the animals with the anti-inflammatory and immunosuppressive drug, dexamethasone. The total number of genes subjected to core analysis by IPA was 37 genes for day 45 PI compared to 900 genes for day 93 PI. This difference in the number of differentially expressed genes per dataset could be attributed to the fact that it was possible to visualize the polypoid masses within the ileocecal region at day 93 PI before dissection for microarray analysis. In contrast, macroscopical lesions were not visible at day 45 PI and the samples were dissected blindly. The core analysis of the datasets focused upon the following modules namely upstream regulators, disease and functions and regulator effects. The aim of IPA upstream regulator analytic is to identify the cascade of upstream transcriptional regulators that can explain the gene expression changes in the experimental datasets and in turn help to clarify the biological activities occurring in the tissue. The top three common upstream regulators (*LPS*, *IFNγ* and *TNF-α*) identified at day 45 PI and day 93 PI, are likely obvious consequences of the presence of *C. parvum* infection in the tissue. Indeed, the prevailing transcriptional response of IECs to *C. parvum* was to upregulate gene targets of *IFNγ* signaling [36]. Moreover, *TNF-α* released from inflammatory monocytes assists *C. parvum* in loss of intestinal barrier to propagate the infection [20]. In parallel, *LPS* from bacteria are known activators of Toll-like receptor 4 (*TLR4*) signaling which is similarly activated for *C. parvum* eradication after activation of the *NF-κβ* signaling pathway [37]. Thus, identification of *LPS* as one of the upstream regulators suggested the presence of still unknown parasitic virulence factors homologous to *LPS*. The top "diseases and disorders" associated with the experimental datasets were "inflammatory response", "immunological disease" and "organismal injury and abnormalities". Moreover, identification of gastrointestinal disease as one of the disorders supports the fact that the differentially expressed genes were detected as a response to *C. parvum*. "Mammalian infection" was the top regulatory pathway identified to be inhibited at day 45 PI and the genes responsible for this action were associated with MHC class II molecules. Antigen presenting cells such as conventional dendritic cells and macrophages found in the intestinal lamina propria have been identified in response to *C. parvum* infection [38]. In IECs, *IFNγ* triggers *CIITA*, which in turn is responsible for the expression of MHC class II molecules [39].

Guanylate binding proteins (*GBP2*, *GBP4*, *GBP6*, *GBP8* and *GBP11*), a superfamily of large GTPases, were found upregulated. It is known that they are induced by *IFNγ* as a host response to external pathogens [22]. Especially *GBP2* has been identified to be recruited by another Apicomplexa, *Toxoplasma gondii* at the host-parasite interface, in the parasitophorous vacuole [40]. Considering, that the parasitophorous vacuole formation is also a hallmark feature of *Cryptosporidium* spp. infection, it may be suggested that upregulation of these genes represents a conserved mechanism of defense among Apicomplexan parasites. Similar observation has also been recorded in another microarray study on *C. parvum* infected intestinal epithelial cells of piglets [36]. On the basis of these data, the *IFNγ*-induced signaling pathway appears to be a highly enriched network on which the host defense mechanism depends.

In parallel to the host defense mechanism aiming at eradicating the infection, the parasite has developed several escape mechanisms to delay these protective mechanisms [38]. Particularly, it has been shown that *C. parvum* can circumvent *CCL20*, a chemokine known to exert anti-microbial activity [41]. Interestingly, this protection during infection was also downregulated at day 93 PI in our study. In addition, defensin-α (*DEFA4*) was highly upregulated at day 45 PI compared to day 93 PI wherein it was highly downregulated. α-defensin is a microbicidal peptide expressed by the Paneth cells to contribute to the innate enteric immunity which has shown parasiticidal activity against *Giardia intestinalis* [42]. Increased expression of LL37 and α-defensin 2 in response to IL18 perturbates intracellular development of *C. parvum* in human cell lines [43]. Thus, it can be proposed that α-defensins are part of the strategy of the host to eradicate *Cryptosporidium* infection even if the parasite seems to be able to downregulate these genes as another immune evasion strategy. Consistently, the downregulation of defensin-β (*DEFB1*) genes in host cells following *C. parvum* infection has been described to be the result of trans-suppression caused by an RNA transcript delivered into the host cell by the parasite [44]. *C. parvum* has been predicted to downregulate the expression of *DEFA4* via the *IGF* signaling pathway at day 93 PI. *IGF* signaling pathway has in turn been predicted to be activated by overexpression of *IGF1*. *IGF-1* signaling pathway was also observed to mediate downregulation of *HPGD* along with other dehydrogenases at day 93 PI, an enzyme responsible for degradation of prostaglandins which are produced by Prostaglandin-Endoperoxide Synthase 2 (*PTGS2*). Prostaglandins are pro-inflammatory lipid mediators which promote cancer cell proliferation, angiogenesis, survival, migration, and invasion [45]. Therefore, persistent *C. parvum* infection might possibly result in the attenuated expression of defensins with deregulation of the host immunity and alteration of the balance towards

inflammation associated cancer. In parallel, loss of the *MCC* gene in a mouse model showed upregulation of *IFNγ*-induced GTPase superfamily in the absence of any external pathogen involvement in the inflamed colon and was restricted to immediate proximity of the damaged epithelial barrier leading to development of cancer [22]. Hence, it can be suggested that intestinal epithelial cells express *IIGP1* as an immune response against *C. parvum* but the parasite would be able to resist to this innate immune response. Then, the overexpression of *IIGP1* would result in chronic inflammation contributing to development of cancer.

The role of chronic cryptosporidiosis in the induction of digestive cancer was further confirmed after the analysis of molecular data at day 93 PI which allowed the identification of genes belonging to five canonical pathways associated with cancer. Moreover, the top disorder associated with this dataset termed as "Organismal Injuries and Abnormalities" was a result of several functions related to cancer progression such as neoplasia, growth, invasion, and metastasis of solid tumor. Identification of 29 out of 173 canonical molecules associated with "Tumor Microenvironment Pathway" predicts that *C. parvum* infection induces a pro-tumorigenic immune response, mediated by diverse immunosuppressive cell signaling molecules resulting in host immune evasion. Considering its over expression, *TNF-α* was identified as a key pro-inflammatory cytokine which stimulate tumor progression at day 93 PI. *TNF-α* produced by inflammatory monocytes assist *C. parvum* to alter the intestinal barrier and the intestinal permeability [20]. In a previous experimental animal infection with *C. parvum,* immunohistochemical abnormal localization of Wnt signaling pathway components together with alterations in the ultrastructure of adherens junctions of the ileo-cecal neoplastic epithelia were recorded [18]. Consequently, in *C. parvum* induced carcinogenesis, *TNF-α* could be an inflammatory stimuli triggering EMT and contributing to lesion development. In addition, expression of molecules involved in ECM remodeling such as several metalloproteinases (*MMP2*, *MMP9*, *MMP10* and *MMP13*), serine protease (*PLAU*) collagens, fibronectins and integrins indicate that functional EMT transition and metastasis take place within the tumor microenvironment [46].

On the other hand, oncogenic pathogens such as *H. pylori* or Epstein Barr virus share common signaling pathways which lead to EMT suggesting that these pathogens may be considered as EMT inducers able to cause a sustained activation of EMT regulating signaling pathways such as NFκβ, MAPK and PI3K/AKT [47]. Consistently, *AKT3*, one of the isoforms of *AKT* that modulates various cellular responses via PI3K/AKT pathway was upregulated at day 93 PI. Interestingly, the over-activation of *AKT* has also been reported in various cancers [48].

Results

Inflammatory stimuli-mediated EMT has been shown to confer immunoregulatory properties to neoplastic epithelial cells by activation of *IDO1* and enhancing tumor immune escape [49]. Indeed, *IDO1* is responsible for catabolism of tryptophan within a tumor microenvironment. Unavailability of the tryptophan arrests T cell proliferation and induces apoptosis which results in immune escape of the cancer cells [50]. In accordance with these observations, we propose herein a gene network in which inflammatory EMT triggered by *IFNγ* and *TNF-α* via *STAT1* results in upregulation of *IDO1* in IECs. In support of this result, *IDO1* expression in Paneth cells has been observed to be strictly regulated by *STAT1* and regarded as an immunosurveillance escape strategy of colorectal cancer [51]. Even though, the downregulation of *IDO1* through the disruption of the *IFNγ* induced *STAT1* pathway has been reported in vitro in early stages of *C. parvum* infection of epithelial cells [52]. Within a tumor microenvironment, high expression of *IDO1* is also indicative of the presence of a population of tumor-infiltrating immune cells such as myeloid-derived suppressor cells (MDSCs) which are known to have suppressive effect on adaptive immune responses [50]. The presence of MDSCs in tumor microenvironment induced by *C. parvum* can also be predicted by the detection of upregulated expression of *ARG1*, another immune suppressive factor which depletes arginine in the microenvironment [50].

Upregulated expression of growth factors such as *IGF1, FGF7*, proteases such as *MMP2*, ECM constituents such as *SPP1* also known as osteopontin and chemokine such as *CXCL12* may indicate the presence of cancer-associated fibroblasts (CAFs) within the tumor microenvironment [53]. *FGF* and *CXCL12* released by CAFs are known to induce tumor growth and angiogenesis. Tumor-associated macrophages (TAMs) polarization represents a key process in tumor progression since TAMs are derived from blood monocytes that can be activated to either M1 (anti-tumor) or M2 (pro-tumor) polarization states depending on the microenvironment stimuli. Within the tumor microenvironment induced by *C. parvum*, upregulated expression of immune suppressive factors such as *ARG1* [54] and *SPP1* [55] probably indicates the presence of tumor promoting M2 TAMs. Inflammation caused by TAMs promotes tumor growth by inducing vascular permeability via upregulation of pro-inflammatory prostaglandins. Upregulated expression of *PTGS2*, previously described to be expressed in IECs can be predicted to increase prostaglandin production and assist TAMs in promoting tumor angiogenesis [56]. *STAT1* and *NF-κβ* have been identified as key transcriptional regulators of *PTGS2* expression, similar to what happens in *H. pylori* infection that induces gastric cancer via chronic inflammation that activates *NF-κβ* which in turn induces

pro-inflammatory mediators such as *IL1, IL6, IL8, TNF-α* and *PTGS2* [57]. *UBD* which also contributes to colon cancer progression [58] was observed to be upregulated in the datasets and its expression was predicted to be induced by *TNF-α* in transformed epithelial cells. Moreover, IECs infected with *C. parvum* showed upregulated expression of *UBD* along with interferon stimulated genes [36]. Thus, it can be speculated that an inflammatory response induces the expression of *UBD* and its role in *C. parvum* associated oncogenesis can be explained by its ability to inhibit tumor suppressor *p53*. Consistently, after infection of animals with *C. parvum* an abnormal localization of p53 protein was found [18].

Even though the majority of the genes required for the maintenance of tumor microenvironment were detected at day 93 PI, some others genes such as *PD-L1*, an immune checkpoint gene, were observed to be exclusively expressed at day 45 PI. Indeed, *PD-L1* which is expressed on tumor cells and infiltrating myeloid cells can inhibit T cell function by inducing apoptosis [59]. Thus, *PD-L1* signaling could be predicted to mediate the contribution of chronic inflammation to carcinogenesis by preventing transformed epithelial cells from $CD8^+$ T-cell attack at day 45 PI [57]. All these data suggests that the development of immunosuppressive tumor microenvironment would be associated with chronic cryptosporidiosis.

Along with all the upregulated genes, the list of several downregulated genes such as cytochrome p450 (*Cyp2c18*, *Cyp2c40*) sulfotransferases (*SULT1C2*), solute carrier proteins (*SLC10a2*, *SLC10A2*) also support the hypothesis of a role of persistent *C. parvum* infection leading to inflammation-assisted cancer progression. Downregulation of these genes was found in DSS-induced colitis model in mice [60] and a coordinated decrease in the expression of the drug response gene cluster was also observed in a mouse model which developed cancer as a result of inflammation-induced DNA damage [22].

5. Conclusions

The data collected herein strongly suggest that chronic inflammation associated with chronic infection plays an important role in *C. parvum*-induced digestive neoplasia. The ability of the parasite to evade the host innate immune response by resisting the upregulated expression of *IFNγ*-stimulated genes and downregulating expression of α-defensins gives rise to the chronic inflammation. Systematic inflammation may contribute to the *C. parvum*-induced immunosuppressive tumor microenvironment. Further studies are needed to understand the immune escape strategies and the pathogenicity of this parasite highly oncogenic when inoculated in an animal model, and to substantiate additional links with cancer development.

Results

Supplementary Materials

The following are available online at www.mdpi.com/article/10.3390/microorganisms9122569/s1, Table S1. Primers used for RT-qPCR. Table S2. Differentially regulated genes at day 45 PI (LogFC2.0; adj *p*-value < 0.05). Table S3. Differentially regulated genes at day 93 PI (LogFC2.0; adj *p*-value < 0.05). References [61,62,63,64] are cited in the Supplementary Materials

Author Contributions

Conceptualization, D.H. and G.C.; funding acquisition, D.H., E.V. and G.C.; investigation, M.S., S.B.-V., A.M. (Anthony Mouray), P.B., N.G., C.C., E.D., A.M. (Adriana Mihalache), P.G., M.C., D.H., E.V. and G.C.; project administration, M.S., S.B.-V., A.M., D.H. and G.C.; resources, M.S. and D.H.; software, M.S. and D.H.; supervision, D.H. and G.C.; validation, D.H., E.V. and G.C.; writing—original draft, M.S. and D.H.; writing—review & editing, M.S., S.B.-V., M.C., D.H., E.V. and G.C. All authors have read and agreed to the published version of the manuscript.

Funding

This study was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Institut Pasteur of Lille, the University of Lille and the Centre Hospitalier Regional Universitaire de Lille (CHU). M.S. was supported by a PhD fellowship from the University of Lille.

Institutional Review Board Statement

Experiments were conducted in the animal facility at the Institute Pasteur of Lille, France (research accreditation number, D 59 350 009). Animal protocols were approved by the French regional ethical committee with the number APAFIS#9621.

Informed Consent Statement

Not applicable.

Data Availability Statement

The data provided in this study has been deposited at NCBI Gene Expression Omnibus (GSE188591) and is available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE188591 (accessed on 12 November 2021).

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ramirez, N.E.; Ward, L.A.; Sreevatsan, S. A Review of the Biology and Epidemiology of Cryptosporidiosis in Humans and an-imals. *Microbes Infect.* **2004**, *6*, 773–785.
2. Kotloff, K.L.; Nataro, J.P.; Blackwelder, W.C.; Nasrin, D.; Farag, T.H.; Panchalingam, S.; Wu, Y.; Sow, S.O.; Sur, D.; Breiman, R.F.; et al. Burden and Aetiology of Diarrhoeal Disease in Infants and Young Children in Developing Countries (The Global Enteric Multicenter Study, GEMS): A Prospective, Case-Control Study. *Lancet* **2013**, *382*, 209–222. https://doi.org/10.1016/s0140-6736(13)60844-2.
3. Khalil, I.A.; Troeger, C.; Rao, P.C.; Blacker, B.F.; Brown, A.; Brewer, T.G.; Colombara, D.V.; De Hostos, E.L.; Engmann, C.; Guerrant, R.L.; et al. Morbidity, Mortality, and Long-Term Consequences Associated with Diarrhoea from Cryptosporidium Infection in Children Younger Than 5 Years: A Meta-Analyzes Study. *Lancet Glob. Health* **2018**, *6*, e758–e768. https://doi.org/10.1016/s2214-109x(18)30283-3.
4. Baldursson, S.; Karanis, P. Waterborne Transmission of Protozoan Parasites: Review of Worldwide Outbreaks—An Update 2004–2010. *Water Res.* **2011**, *45*, 6603–6614.
5. Efstratiou, A.; Ongerth, J.E.; Karanis, P. Waterborne Transmission of Protozoan Parasites: Review of Worldwide Outbreaks—An Update 2011–2016. *Water Res.* **2017**, *114*, 14–22.
6. CDC Crypto Outbreaks Linked to Swimming Have Doubled since 2014. Available online: https://www.cdc.gov/media/releases/2017/p0518-Cryptosporidium-outbreaks.html (accessed on 1 September 2021).
7. Okhuysen, P.C.; Chappell, C.L.; Crabb, J.H.; Sterling, C.R.; Dupont, H.L. Virulence of Three Distinct *Cryptosporidium parvum* Isolates for Healthy Adults. *J. Infect. Dis.* **1999**, *180*, 1275–1281. https://doi.org/10.1086/315033.
8. Rowan, N.J. Defining Established and Emerging Microbial Risks in the Aquatic Environment: Current Knowledge, Implications, and Outlooks. *Int. J. Microbiol.* **2010**, *2011*, 1–15. https://doi.org/10.1155/2011/462832.
9. Yoder, J.S.; Beach, M.J. Cryptosporidium Surveillance and Risk Factors in the United States. *Exp. Parasitol.* **2010**, *124*, 31–39. https://doi.org/10.1016/j.exppara.2009.09.020.
10. Gargala, G. Drug Treatment and Novel Drug Target against Cryptosporidium. *Parasite* **2008**, *15*, 275–281. https://doi.org/10.1051/parasite/2008153275.

11. Osman, M.; Benamrouz, S.; Guyot, K.; Baydoun, M.; Fréalle, E.; Chabé, M.; Gantois, N.; Delaire, B.; Goffard, A.; Aoun, A.; et al. High Association of Cryptosporidium spp. Infection with Colon Adenocarcinoma in Lebanese patients. *PLoS ONE* **2017**, *12*, e0189422. https://doi.org/10.1371/journal.pone.0189422.
12. Zhang, N.; Yu, X.; Zhang, H.; Cui, L.; Li, X.; Zhang, X.; Gong, P.; Li, J.; Li, Z.; Wang, X.; et al. Prevalence and Genotyping of Cryptosporidium parvum in Gastrointestinal Cancer Patients. *J. Cancer* **2020**, *11*, 3334–3339. https://doi.org/10.7150/jca.42393.
13. Sulżyc-Bielicka, V.; Kołodziejczyk, L.; Jaczewska, S.; Bielicki, D.; Safranow, K.; Bielicki, P.; Kładny, J.; Rogowski, W. Colorectal Cancer and Cryptosporidium spp. Infection. *PLoS ONE* **2018**, *13*, e0195834. https://doi.org/10.1371/journal.pone.0195834.
14. Kopacz, Ż.; Kváč, M.; Karpiński, P.; Hendrich, A.B.; Sąsiadek, M.M.; Leszczyński, P.; Sak, B.; McEvoy, J.; Kicia, M. The First Evidence of Cryptosporidium meleagridis Infection in a Colon Adenocarcinoma from an Immunocompetent Patient. *Front. Cell Infect. Microbiol.* **2019**, *9*, 35.
15. Shebl, F.M.; Engels, E.A.; Goedert, J.J. Opportunistic Intestinal Infections and Risk of Colorectal Cancer Among People with AIDS. *AIDS Res. Hum. Retrovir.* **2012**, *28*, 994–999. https://doi.org/10.1089/aid.2011.0185.
16. Certad, G.; Ngouanesavanh, T.; Guyot, K.; Gantois, N.; Chassat, T.; Mouray, A.; Fleurisse, L.; Pinon, A.; Cailliez, J.-C.; Dei-Cas, E.; et al. Cryptosporidium parvum, a Potential Cause of Colic Adenocarcinoma. *Infect. Agents Cancer* **2007**, *2*, 22. https://doi.org/10.1186/1750-9378-2-22.
17. Benamrouz, S.; Guyot, K.; Gazzola, S.; Mouray, A.; Chassat, T.; Delaire, B.; Chabé, M.; Gosset, P.; Viscogliosi, E.; Dei-Cas, E.; et al. Cryptosporidium parvum Infection in SCID Mice Infected with Only One Oocyst: qPCR Assessment of Parasite Replication in Tissues and Development of Digestive Cancer. *PLoS ONE* **2012**, *7*, e51232. https://doi.org/10.1371/journal.pone.0051232.
18. Benamrouz, S.; Conseil, V.; Chabé, M.; Praet, M.; Audebert, C.; Blervaque, R.; Guyot, K.; Gazzola, S.; Mouray, A.; Chassat, T.; et al. Cryptosporidium parvum-Induced Ileo-Caecal Adenocarcinoma and Wnt Signaling in a Mouse Model. *Dis. Model. Mech.* **2014**, *7*, 693–700.
19. Baydoun, M.; Vanneste, S.B.; Creusy, C.; Guyot, K.; Gantois, N.; Chabe, M.; Delaire, B.; Mouray, A.; Baydoun, A.; Forzy, G.; et al. Three-Dimensional (3D) Culture of Adult Murine Colon as an In Vitro Model of Cryptosporidiosis: Proof of Concept. *Sci. Rep.* **2017**, *7*, 17288. https://doi.org/10.1038/s41598-017-17304-2.

20. Sablet, T.; Potiron, L.; Marquis, M.; Bussiere, F.I.; Lacroix-Lamande, S.; Laurent, F. *Cryptosporidium parvum* Increases the in-Testinal Permeability through Interaction with Epithelial Cells and IL-1β and TNF-α Released by Inflammatory Monocytes. *Cell Microbiol.* **2016**, *18*, 1871–1880.
21. Chen, X.M.; Levine, S.A.; Splinter, P.L.; Tietz, P.S.; Ganong, A.L.; Jobin, C.; Gores, G.J.; Paya, C.V.; LaRusso, N.F. Cryp-Tosporidium Parvum Activates Nuclear Factor kappaB in Biliary Epithelia Preventing Epithelial Cell Apoptosis. *Gastroenterology* **2001**, *120*, 1774–1783.
22. Currey, N.; Jahan, Z.; Caldon, C.E.; Tran, P.N.; Benthani, F.; De Lacavalerie, P.; Roden, D.L.; Gloss, B.S.; Campos, C.; Bean, E.G.; et al. Mouse Model of Mutated in Colorectal Cancer Gene Deletion Reveals Novel Pathways in Inflammation and Cancer. *Cell. Mol. Gastroenterol. Hepatol.* **2019**, *7*, 819–839. https://doi.org/10.1016/j.jcmgh.2019.01.009.
23. Liu, T.L.; Fan, X.C.; Li, Y.H.; Yuan, Y.J.; Yin, Y.L.; Wang, X.T.; Zhang, L.X.; Zhao, G.H. Expression Profiles of mRNA and IncRNA in HCT-8 Cells Infected with *Cryptosporidium parvum* IId Subtype. *Front. Microbiol.* **2018**, *9*, 1409
24. He, W.; Li, J.; Gong, A.-Y.; Deng, S.; Li, M.; Wang, Y.; Mathy, N.W.; Feng, Y.; Xiao, L.; Chen, X.M. Cryptosporidial Infection Suppresses Intestinal Epithelial Cell MAPK Signaling Imparing Host Anti-Parasitic Defense. *Microorganisms* **2021**, *9*, 151.
25. Mary, C.; Chapey, E.; Dutoit, E.; Guyot, K.; Hasseine, L.; Jeddi, F.; Menotti, J.; Paraud, C.; Pomares, C.; Rabodonirina, M.; et al. Multicentric Evaluation of a New Real-Time PCR Assay for Quantification of Cryptosporidium spp. and Identification of *Cryptosporidium parvum* and Cryptosporidium hominis. *J. Clin. Microbiol.* **2013**, *51*, 2556–2563. https://doi.org/10.1128/jcm.03458-12.
26. Boivin, G.P.; Washington, K.; Yang, K.; Ward, J.M.; Pretlow, T.P.; Russell, R.; Besselsen, D.G.; Godfrey, V.L.; Doetschman, T.; Dove, W.F.; et al. Pathology of Mouse Models of Intestinal Cancer: Consensus Report and Recommendations. *Gastroenterology* **2003**, *124*, 762–777. https://doi.org/10.1053/gast.2003.50094.
27. Certad, G.; Creusy, C.; Ngouanesavanh, T.; Guyot, K.; Gantois, N.; Mouray, A.; Chassat, T.; Flament, N.; Fleurisse, L.; Pinon, A.; et al. Development of Cryptosporidium parvum-Induced Gastrointestinal Neoplasia in Severe Combined Immunodeficiency (SCID) Mice: Severity of Lesions Is Correlated with Infection Intensity. *Am. J. Trop. Med. Hyg.* **2010**, *82*, 257–265. https://doi.org/10.4269/ajtmh.2010.09-0309.

28. Stolte, M. The New Vienna Classification of Epithelial Neoplasia of the Gastrointestinal Tract: Advantages and Disadvantages. **2003**, *442*, 99–106. https://doi.org/10.1007/s00428-002-0680-3.
29. Smyth, G.K.; Yang, Y.H.; Speed, T.; Brownstein, M.J.; Khodursky, A. Statistical Issues in cDNA Microarray Data Analysis. *Methods Mol. Biol.* **2003**, *224*, 111–136. https://doi.org/10.1385/1-59259-364-x:111.
30. Ihaka, R.; Gentleman, R. A Language for Data Analysis and Graphics. *J. Comput. Graph. Statistics.* **1996**, *5*, 299–314.
31. Yang, Y.H.; Dudoit, S.; Luu, P.; Lin, D.M.; Peng, V.; Ngai, J.; Speed, T.P. Normalization for cDNA Microarray Data: A Robust Composite Method Addressing Single and Multiple Slide Systematic Variation. *Nucleic Acids Res.* **2002**, *30*, e15.
32. Lonnstedt, I.; Speed, T. Replicated Microarray Data. *Stat. Sin.* **2002**, *12*, 31–46.
33. Thomas, S.; Bonchev, D. A Survey of Current Software for Network Analysis in Molecular Biology. *Hum. Genom.* **2010**, *4*, 353–360. https://doi.org/10.1186/1479-7364-4-5-353.
34. Kramer, A.; Green, J.; Pollard, J.; Tugendreich, S. Casual Analysis Approaches in Ingenuity Pathway Analysis. *Bioinformatics* **2014**, *30*, 523–530.
35. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(−ΔΔC(T)) Method. *Methods* **2001**, *25*, 402–408
36. Ferguson, S.H.; Foster, D.; Sherry, B.; Magness, S.T.; Nielsen, D.M.; Gookin, J.L. Interferon-λ3 Promotes Epithelial Defense and Barrier Function against *Cryptosporidium parvum* Infection. *Cell. Mol. Gastroenterol. Hepatol.* **2019**, *8*, 1–20. https://doi.org/10.1016/j.jcmgh.2019.02.007.
37. O'Hara, S.P.; Chen, X.-M. The Cell Biology of Cryptosporidium Infection. *Microbes Infect.* **2011**, *13*, 721–730. https://doi.org/10.1016/j.micinf.2011.03.008.
38. Laurent, F.; Lacroix-Lamandé, S. Innate Immune Responses Play a Key Role in Controlling Infection of the Intestinal Epithelium by Cryptosporidium. *Int. J. Parasitol.* **2017**, *47*, 711–721. https://doi.org/10.1016/j.ijpara.2017.08.001.
39. Heuberger, C.; Pott, J.; Maloy, K.J. Why Do Intestinal Epithelial Cells Express MHC Class II? *Immunology* **2021**, *162*, 357–367.
40. Foltz, C.; Napolitano, A.; Khan, R.; Clough, B.; Hirst, E.M.; Frickel, E.M. TRIM21 Is Critical for Survival of *Toxoplasma gondii* Infection and Localizes to GBP-Positive Parasite Vacuoles. *Sci. Rep.* **2017**, *7*, 5209.

41. Guesdon, W.; Auray, G.; Pezier, T.; Bussière, F.I.; Drouet, F.; Le Vern, Y.; Marquis, M.; Potiron, L.; Rabot, S.; Bruneau, A.; et al. CCL20 Displays Antimicrobial Activity against Cryptosporidium parvum, but Its Expression Is Reduced During Infection in the Intestine of Neonatal Mice. *J. Infect. Dis.* **2015**, *212*, 1332–1340. https://doi.org/10.1093/infdis/jiv206.
42. Aley, S.B.; Zimmerman, M.; Hetsko, M.; Selsted, M.E.; Gillin, F.D. Killing of Giardia Lamblia by Cryptdins and Cationic Neu-trophil Peptides. *Infect. Immun.* **1994**, *62*, 5397–5403.
43. McDonald, V.; Pollok, R.C.G.; Dhaliwal, W.; Naik, S.; Farthing, M.J.G.; Bajaj-Elliott, M. A Potential Role for Interleukin-18 in Inhibition of the Development of Cryptosporidium parvum. *Clin. Exp. Immunol.* **2006**, *145*, 555–562. https://doi.org/10.1111/j.1365-2249.2006.03159.x.
44. Ming, Z.; Gong, A.-Y.; Wang, Y.; Zhang, X.-T.; Li, M.; Dolata, C.E.; Chen, X.-M. Trans-Suppression of Defense DEFB1 Gene in Intestinal Epithelial Cells Following *Cryptosporidium parvum* Infection Is Associated with Host Delivery of Parasite Cdg7_FLc_1000 RNA. *Parasitol. Res.* **2018**, *117*, 831–840. https://doi.org/10.1007/s00436-018-5759-0.
45. Wang, D.; Dubois, R.N. Role of Prostanoids in Gastrointestinal Cancer. *J. Clin. Investig.* **2018**, *128*, 2732–2742. https://doi.org/10.1172/jci97953.
46. Fu, Y.; Liu, S.; Zeng, S.; Shen, H. From Bench to Bed: The Tumor Immune Microenvironment and Current Immunotherapeutic Strategies for Hepatocellular Carcinoma. *J. Exp. Clin. Cancer Res.* **2019**, *38*, 396. https://doi.org/10.1186/s13046-019-1396-4.
47. Hofman, P.; Vouret-Craviari, V. Microbes-Induced EMT at the Crossroad of Inflammation and Cancer. *Gut Microbes* **2012**, *3*, 176–185. https://doi.org/10.4161/gmic.20288.
48. Takahashi, H.; Rokudai, S.; Kawabata-Iwakawa, R.; Sakakura, K.; Oyama, T.; Nishiyama, M.; Chikamatsu, K. AKT3 Is a Novel Regulator of Cancer-Associated Fibroblasts in Head and Neck Squamous Cell Carcinoma. *Cancers* **2021**, *13*, 1233. https://doi.org/10.3390/cancers13061233.
49. Ricciardi, M.; Zanotto, M.; Malpeli, G.; Bassi, G.; Perbellini, O.; Chilosi, M.; Bifari, F.; Krampera, M. Epithelial-to-Mesenchymal Transition (EMT) Induced by Inflammatory Priming Elicits Mesenchymal Stromal Cell-like Immune-Modulatory Properties in Cancer Cells. *Br. J. Cancer* **2015**, *112*, 1067–1075. https://doi.org/10.1038/bjc.2015.29.

50. Nagaraj, S.; Gabrilovich, D.I. Tumor Escape Mechanism Governed by Myeloid-Derived Suppressor Cells. *Cancer Res.* **2008**, *68*, 2561–2563. https://doi.org/10.1158/0008-5472.can-07-6229.
51. Pflügler, S.; Svinka, J.; Scharf, I.; Crncec, I.; Filipits, M.; Charoentong, P.; Tschurtschenthaler, M.; Kenner, L.; Awad, M.; Stift, J.; et al. IDO1+ Paneth Cells Promote Immune Escape of Colorectal Cancer. *Commun. Biol.* **2020**, *3*, 252. https://doi.org/10.1038/s42003-020-0989-y.
52. Choudhry, N.; Korbel, D.S.; Edwards, L.A.; Bajaj-Elliott, M.; McDonald, V. Dysregulation of Interferon-Gamma-Mediated Signaling Pathway in Intestinal Epithelial Cells by *Cryptosporidium parvum* Infection. *Cell. Microbiol.* **2009**, *11*, 1354–1364.
53. Peddareddigari, V.G.; Wang, D.; DuBois, R.N. The Tumor Microenvironment in Colorectal Carcinogenesis. *Cancer Microenviron.* **2010**, *3*, 149–166. https://doi.org/10.1007/s12307-010-0038-3.
54. Solinas, G.; Germano, G.; Mantovani, A.; Allavena, P. Tumor-Associated Macrophages (TAM) as Major Players of the Cancer-Related Inflammation. *J. Leukoc. Biol.* **2009**, *86*, 1065–1073.
55. Shurin, M.R. Osteopontin Controls Immunosuppression in the Tumor Microenvironment. *J. Clin. Investig.* **2018**, *128*, 5209–5212. https://doi.org/10.1172/jci124918.
56. Eberhart, C.E.; Coffey, R.J.; Radhika, A.; Giardiello, F.M.; Ferrenbach, S.; DuBois, R.N. Up-Regulation of Cyclooxygenase 2 Gene Expression in Human Colorectal Adenomas and Adenocarcinomas. *Gastroenterology* **1994**, *107*, 1183–1188.
57. Wang, D.; Dubois, R.N. Immunosuppression Associated with Chronic Inflammation in the Tumor Microenvironment. *Carcinogenesis* **2015**, *36*, 1085–1093. https://doi.org/10.1093/carcin/bgv123.
58. Yan, D.-W.; Li, D.-W.; Yang, Y.-X.; Xia, J.; Wang, X.-L.; Zhou, C.-Z.; Fan, J.-W.; Wen, Y.-G.; Sun, H.-C.; Wang, Q.; et al. Ubiquitin D Is Correlated with Colon Cancer Progression and Predicts Recurrence for Stage II-III Disease after Curative Surgery. *Br. J. Cancer* **2010**, *103*, 961–969. https://doi.org/10.1038/sj.bjc.6605870.
59. Barriga, V.; Kuol, N.; Nurgali, K.; Apostolopoulos, V. The Complex Interaction between the Tumor Micro-Environment and Immune Checkpoints in Breast Cancer. *Cancers* **2019**, *11*, 1205. https://doi.org/10.3390/cancers11081205.
60. Fang, K.; Bruce, M.; Pattillo, C.B.; Zhang, S.; Stone, R.; Clifford, J.; Kevil, C.G. Temporal Genome Wide Expression Profiling of DSS Colitis Reveals Novel Inflammatory and Angiogenesis Genes Similar to Ulcerative Colitis. *Physiol. Genom.* **2011**, *43*, 43–56

3.3. Investigation of signaling pathways at the intersection of *C. parvum* infection and cancer onset

Title: **The role of PI3K/AKT signaling pathway in *C. parvum*-induced digestive neoplasia**

This article is in preparation.

Manasi Sawant, Colette Creusy, Erika Duval, Eric Viscogliosi, Sadia Benamrouz-Vanneste, Gabriela Certad

My contribution to the study:

- Conceptualization of experiments
- Performing the experiments
- Analysis of the data
- Writing the manuscript

Infectious agents including viruses, bacteria, and parasites have been identified as risk factors for certain types of cancer. Considering that PI3K/AKT is one of the most important pathways altered in case of cancers of infectious origin, and that PI3K is an integral molecule involved in the process of cellular invasion of *C. parvum,* we decided to clarify whether PI3K/AKT signaling is activated during *C. parvum*-induced digestive neoplasia. Modulated genes at 93 days post-infection from the dataset corresponding to my article: "Persistent *Cryptosporidium parvum* infection leads to the development of the tumor microenvironment in an experimental mouse model: results of a microarray approach" were analyzed employing the g-Profiler for the identification of KEGG pathways. STRING database (STRING v11.0) was used to predict the protein-protein interactions between the differentially expressed genes in the pathways of interests. Western blotting, and immunofluorescence were used to compare the expression levels of PI3K/AKT signal pathway-related proteins in the epithelia of infected animals compared to non-infected ones. Obtained results showed differences in the expression of proteins involved in PI3K/AKT signaling between *C. parvum* infected and non-infected animals. In conclusion, this study showcases a potential role of PI3K/AKT signaling pathway in *C. parvum* induced carcinogenesis but further studies have to be done to confirm and better understand this mechanism.

Results

Article 3

The role of PI3K/AKT signaling pathway in *C. parvum*-induced digestive neoplasia

Manasi Sawant[1], Colette Creusy[2], Erika Duval[2], Eric Viscogliosi[1], Sadia Benamrouz-Vanneste[1,3], Gabriela Certad[1,4].

[1]Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France

[2] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France

[3]Equipe Ecologie et Biodiversité, FGES, Université Catholique de Lille, F-59000 Lille, France.

[4] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France

Corresponding author: Gabriela Certad

Abstract

Cryptosporidium is a protozoan parasite considered as a leading cause of diarrhea in children and immunocompromised individuals. It has been reported that *C. parvum*, one of the two most important species causing human cryptosporidiosis, is able to induce colic invasive adenocarcinomas in an immunosuppressed mouse model. Considering that PI3K/AKT is one of the most important pathways altered in case of cancer, especially in colorectal cancer and that PI3K is an integral molecule involved in the process of cellular invasion of *C. parvum* we decided to study the role of this signaling pathway in the process of *C. parvum*-induced digestive neoplasia. Kyoto Encyclopedia of Genes and Genomes (KEGG) and STRING Pathway Enrichment Analyzes, western blotting, and immunofluorescence were used. Obtained results showed differences in the expression of proteins involved in PI3K/AKT signaling between *C. parvum* infected and non-infected animals. In conclusion, this study showcases a potential role of PI3K/AKT signaling pathway in *C. parvum* induced carcinogenesis but further studies have to be done to confirm and better understand this mechanism.

Results

Introduction

Cryptosporidium is a protozoan parasite considered as a leading cause of diarrhea in children and immunocompromised individuals. Moreover, it has been reported that *C. parvum*, one of the two most important species causing human cryptosporidiosis, is able to induce colic invasive adenocarcinomas in an immunosuppressed mouse model (1). Alterations in cellular expression of APC, β-catenin and p53 proteins were reported. The cytoskeletal reorganization has also been suggested to play a critical role in the *C. parvum* induced cell transformation based on the observation of dilation of intercellular spaces with extensive development of lateral membrane extensions in the parasite infected epithelial cells showing neoplasia. (2). One important signaling kinase implicated in actin polymerization and activated upon membrane stimulation by a variety of ligands is the class IA phosphatidylinositol 3-kinase (PI3K) (3). Furthermore, PI3K/AKT pathway is one of the most important pathways dysregulated in case of cancer, especially colorectal cancer (4), and this is frequently associated with a dysregulation of EGFR/MAPK pathway (5). In the case of *C. parvum*-induced neoplasia, preliminary biochemical study performed on ileo-cecal region of SCID mice infected with the parasite, targeting ERK (Extracellular signal-regulated kinase), showed that there is no difference in the ERK expression or activation (unpublished data). Interestingly, PI3K/AKT is known to phosphorylate IκB kinase (IKK) which regulate positively NF-κB (Nuclear Factor κB) and then promotes cell survival and proliferation (5). Adding to that, the development of *Cryptosporidium* cycle, requires modulation of apoptosis which turns to be biphasic. Indeed, during the multiplication phase, the apoptosis of the host cell is inhibited whereas after oocyst formation the apoptosis is restored (6). The mechanisms for this regulation are still poorly understood, but it seems that the NF-κB pathway is activated in host cells and leads to inhibition of apoptosis (7). It has been also shown in human biliary epithelial infected cells that *C. parvum* induces the accumulation of PI3K at the host-cell parasite interface resulting in activation of PI3K. Thus, PI3K is an integral molecule involved in the process of cellular invasion of *C. parvum* (8). Moreover, the regulatory subunit p85α of the PI3K complex is known to be activated during the invasion of host cell by *C. parvum* (8).

Indeed, PI3K is a heterodimeric lipid kinase consisting of the p110α/β catalytic subunit and the p85α/β regulatory subunit. When p85 binds to the phosphorylated tyrosine residues located at the intracellular part of receptor tyrosine kinases (RTKs), the inhibitory effect of p85 on p110 is released and PI3K is activated (3). The PI3K/AKT signaling pathway is activated by many types of cellular stimuli or toxic insults. The activation of PI3K is associated with cell

proliferative, growth and survival signals in epithelial cells. PI3K phosphorylates PIP2 to generate PIP3, which functions as a ligand to recruit pleckstrin homology (PH) domain-containing proteins to the inner surface of the cellular membrane. Chief among these is AKT a serine/threonine kinase that is activated by phosphorylation at Ser 473 and Thr 308 by PDK1 and PDK2. AKT has emerged as a critical signaling node in mammalian cells and mediates the majority of PI3K's downstream effects. More importantly, AKT triggers a cascade of responses, from cell growth and proliferation to survival and motility, which drive tumor progression (9) (Figure 1).

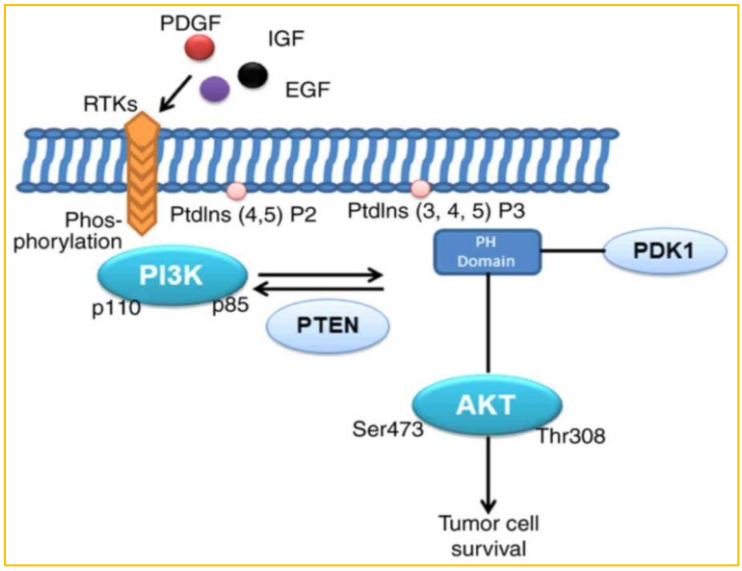


Figure 1. Schematic representation of the PI3K/AKT signaling pathway. RTKs recruit PI3K following phosphorylation and activated PI3K phosphorylates PtdIns P2 (PIP2) to PtdIns P3 (PIP3) which in turn recruits PDK1 to the PH domain of AKT, there by activating the entire pathway and regulating cell growth. PI3K, phosphatidylinositol 3-linase; AKT, protein kinase B; RTK, receptor tyrosine kinase; PtdIns, phosphatidylinositol; PDK1, 3-phosphoinositol-dependent protein kinase 1; PH, plekstrin homology; PTEN, phosphatase and tensin homolog; PDGF, platelet derived growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor. (Image adapted from (Shi X et al. 2019)).

For all these reasons, we hypothesized that the PI3K/AKT signaling pathway maybe involved in the process of *C. parvum* induced carcinogenesis. In order to test this hypothesis, we decided in this study to assess the regulation of activations of the two most important proteins of this pathway, which are PI3K and AKT.

Results

Material and methods

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyzes

Modulated genes at 93 days post-infection from the dataset corresponding to (10) (https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE188591) were analyzed employing the g-Profiler for the identification of KEGG pathways. STRING database (STRING v11.0) was used to predict the protein-protein interactions between the differentially expressed genes in the pathways of interest (http://string-db.org/).

Cryptosporidium Oocysts

C. parvum strain IOWA oocysts were purchased from Waterborne, Inc. (New Orleans, LA, USA) and stored in phosphate-buffered saline (PBS) supplemented with penicillin, streptomycin, gentamycin, amphotericin B and 0.001% Tween 20 at 4 °C until use. Absence of bacteria and fungi was assured by testing the oocyst suspensions on Plate Count Agar and Sabouraud plates for 1 week at 37 °C. The oocyst viability was assessed as described previously (10).

Mouse Model of Cryptosporidiosis

Seven-week-old CB17-SCID mice were obtained from a colony bred and regularly controlled for infections (including *Helicobacter* spp.) at the Pasteur Institute of Lille (France). Animals were maintained under aseptic conditions in an isolator during the whole experimentation, in a 12 h light-dark cycle with water and a standard diet (65% carbohydrate, 11% fat, and 24% protein; SAFE, Augy, France). Mice were administered with 4 mg/L of dexamethasone (Merck, Lyon, France) through drinking water. Dexamethasone treatment started 2 weeks prior to inoculation with the parasite and maintained during the entire experimentation. Dexamethasone-containing water was replaced three times a week. Infective doses of *C. parvum* (10^4 oocysts/mouse) were prepared and inoculated by intra-gastric feeding. Control animals were inoculated with PBS. Assessment of the clinical conditions of the mice was performed regularly to detect and then minimize suffering. Mice were euthanized by carbon dioxide inhalation at different time points (day 60) or when clinical signs constitutive of an end point appeared. Samples from the ileo-cecal region were collected. Experiments were conducted in the animal facility at the Institute Pasteur of Lille (research accreditation number, D 59 350 009). Animal protocols were approved by the French regional ethical committee with the number APAFIS#9621.

Results

Oocyst Shedding

The oocyst shedding was evaluated by collecting freshly excreted fecal pellets periodically. Total genomic DNA was extracted from 200 mg of feces by using the QIAamp Fast DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. The presence of parasite was detected by using the TaqMan real time PCR (qPCR) assay as previously described (11).

Histological Examination

Ileo-cecal regions of mice were collected then fixed in 4% buffered formalin. Formalin-fixed and paraffin-embedded specimens were sectioned at a thickened of 5 µm, stained with hematoxylin, eosin and saffron and examined microscopically for the detection of histological modifications of the host tissue. Pathological changes found in the mouse caecum were classified according to the Vienna classification of tumors of the digestive system in humans taking into account the nomenclature for histological assessment of intestinal tumors in rodent models as previously described (11).

Immunofluorescence analysis

Paraffin embedded tissue sections were deparaffinized by serial washes with xylene and rehydrated by immersion through serial dilutions of alcohol. Further, the slides were immersed in 3% hydrogen peroxide solution and boiled at 900 W in microwave for antigen retrieval. After cooling the slides, the sections were blocked with 2.5 % BSA solution. The primary antibodies, anti-p85α rabbit polyclonal antibody (1 :100), a monoclonal anti pAKT (ser 473 phosphorylated) (1:100) and a monoclonal antibody anti-AKT (1:100), diluted in 0,1% Triton X-100 in 1X PBS (Cell Signaling Technology), were applied overnight at 4 °C in humidified chamber. The primary antibody were washed with 0.1% tween-20 in 1x PBS solution for 5 minutes twice. For pAKT, the antigen retrieval buffer was replaced with 0.05% Tween 20 containing 10 mM citrate buffer. The sections were incubated with the secondary antibodyAlexa-488 conjugated anti-rabbit goat antibody (1 :1000) for 1h at room temperature. After washing the secondary antibody, the sections were incubated for 15 minutes in DAPI solution (1µg/µl). Finally the sections were mounted using mowiol solution. The images were acquired using a Zeiss LSM880 confocal microscope and images analyzed using the ZEN lite Digital Imaging Software.

Results

Western blotting analysis

Ilea cecal regions were collected and frozen in liquid nitrogen and stored at -80 °C. Total cellular protein was extracted using standard RIPA buffer supplemented with proteases and phosphatases inhibitors. Proteins were quantified using BCA assay. Total of 10 µg/µl of protein was loaded onto the SDS-PAGE and western blotting was performed. Beta-actin was used as a protein loading control.

Immunoprecipitation and detection of phosphorylated-p85α

20 µl of Sera-Mag Speed Beads protein A/G magnetic particles slurry was washed with 500 µl of 1X RIPA buffer and regarded as pre-washed magnetic beads. Total cellular protein were pre-cleared by incubating with 20 µl of pre-washed magnetic beads for 1h at 4º C. The pre-cleared cellular proteins were incubated overnight with primary antibody (1mg of protein/10 µl of anti-p85α antibody) at 4º C. Then, 20 µl of pre-washed magnetic beads were added to the immunocomplex solution and incubated for 30 minutes at room temperature. The magnetic beads were pelleted using magnetic separation rack. The bead pellet was washed with 500 µl of 1X RIPA buffer four times. The bound proteins were eluted by boiling in 2X SDS sample buffer at 95º C for 5 minutes. The beads were again pelleted using magnetic separation rack. The supernatant was the sample loaded on the SDS-PAGE gel. And immunoblots were subjected to anti-phosphotyrosine antibody (1:1000) (Thermofischer Scientific). Phosphorylated-p85α was detected as a result of immunoreactive bands developed using super signal west femto maximum sensitivity substrate (Thermo Scientific, USA).

Immunofluorescence signal quantification and statistical analysis

Fluorescence intensity signals between infected and non-infected host tissue were quantified using ImageJ software version 1.52a (NIH, USA). For the statistical analysis, a mixed model was used to test the relationship between fluorescence intensity markers and group condition taking in account sample repetition. A mixed regression model was created considering fluorescence quantification as the main outcomes and sample identifier as random effect. The general significance level was set at a p-value below 0.05. All analyzes were performed using packages nlme from the R statistical computing program.

Results

Results

Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analyzes

KEGG analysis was performed on 533 putative modulated targets to determine the relative significance. The top 13 targets are listed in (Table 1). The results show pathway enrichment for the KEGG terms "Chemokine signaling pathway", "Focal Adhesion" and "PI3K/AKT signaling pathway". Base on the STRING database, the interaction networks were constructed for differentially expressed genes of these pathways. The average local clustering coefficient was 0.9 and the protein-protein interaction enrichment P value was 6X10-12 which demonstrated significant interactions among proteins and biological connections. Biological functions of this network were associated to regulate TOR signaling, regulation of signal transduction, regulation of apoptosis. Molecular functions were associated to protein serine/threonine phosphatase activity, protein kinase binding beta-catenin binding (Figure 2).

Table 1. Significantly enriched KEGG pathways according to G. profiler

Signaling Pathway	Adjusted_*P*_value	Observed gene count	Intersections
Cytokine-cytokine receptor interaction	2.6X10 $^{-12}$	46/252	TNF,CXCL1,IL1B,INHBA,IL11,IL10RA,IL34,IL1RL1,TNFSF9 ,CSF2RB2,TNFRSF11B,CCL4,IL1R2,CCL3,IL1RN,CXCL16,C SF2RB,CSF1R,OSMR,IL3RA,IL17RE,CXCL12,IL33,CXCL14, CCL11,TNFRSF1B,PRLR,IL18,BMP4,CCL2,CXCL2,CCL7,TN FRSF12A,TGFBR2,CXCL10,IL6,TNFSF13B,CCL9,INHBB,CC L8,CCL28,CXCL9,CXCR2,CCL12,CXCL13,CXCL5
Viral protein interaction with cytokine and cytokine receptor	1..4X10^{-11}	25/91	TNF,CXCL1,IL10RA,IL34,CCL4,CCL3,CSF1R,CXCL12,CXCL 14,CCL11,TNFRSF1B,IL18,CCL2,CXCL2,CCL7,CXCL10,IL6, CCL9,CCL8,CCL28,CXCL9,CXCR2,CCL12,CXCL13,CXCL5
IL-17 signaling pathway	3.9X $^{10-8}$	21/91	TNF,CXCL1,IL1B,S100A8,S100A9,PTGS2,IL17RE,MMP9, MMP13,CCL11,MMP3,CCL2,CXCL2,CCL7,CXCL10,IL6,CE BPB,LCN2,CCL12,CXCL5,FOSL1
ECM-receptor interaction	**1.7X10^{-7}**	**20/81**	**TNF,CXCL1,IL1B,S100A8,S100A9,PTGS2,IL17RE,MMP9, MMP13,CCL11,MMP3,CCL2,CXCL2,CCL7,CXCL10,IL6,CE BPB,LCN2,CCL12,CXCL5,FOSL1**
Amoebiasis	<0.0001	20/105	NF,CXCL1,ARG1,IL1B,COL1A2,COL1A1,LAMC2,CD14,IL1 R2,LAMA5,LAMC1,LAMA3,COL3A1,ITGB2,LAMB1,COL4 A1,COL4A2,CXCL2,IL6,FN1
Chemokine signaling pathway	**<0.0001**	**26/179**	**CXCL1,HCK,NCF1,CCL4,CCL3,CXCL16,AKT3,CXCL12,CXC L14,VAV3,CCL11,RAC2,STAT1,CCL2,CXCL2,CCL7,CXCL1 0,CCL9,VAV1,CCL8,CCL28,CXCL9,CXCR2,CCL12,CXCL13, CXCL5**
Focal adhesion	**<0.0001**	**27/195**	**COL6A5,COL1A2,COL1A1,LAMC2,IGF1,SPP1,MYL7,COL 2A1,AKT3,LAMA5,LAMC1,LAMA3,COL6A3,VAV3,RAC2 ,LAMB1,COL4A1,THBS1,TNC,ITGB8,PDGFRA,COL4A2,I TGB3,FN1,VAV1,COL6A1,IBSP**
Chemical carcinogenesis - DNA adducts	0.0001	16/92	PTGS2,GSTM6,UGT2B5,UGT1A6B,CYP1B1,UGT2B37,U GT2B38,SULT1A1,MGST1,UGT2B36,HSD11B1,CYP2C55, CYP2C29,UGT2B34,CYP2C40,CYP2C65

TNF signaling pathway	0.0002	18/108	TNF,CXCL1,IL1B,VCAM1,PTGS2,AKT3,MMP9,TNFRSF1B,MMP3,CCL2,ICAM1,CXCL2,CXCL10,IL6,IFI47,CEBPB,CCL12,CXCL5
Leukocyte transendothelial migration	0.002	17/115	NCF4,VCAM1,MYL7,NCF1,ITGB2,JAM2,CYBB,CXCL12,MMP9,MMP2,CDH5,VAV3,CLDN8,RAC2,MSN,ICAM1,VAV1
PI3K/AKT signaling pathway	**0.003**	**34/349**	**COL6A5,COL1A2,COL1A1,PCK1,LAMC2,IGF1,SPP1,COL2A1,AKT3,CSF1R,LAMA5,OSMR,IL3RA,LAMC1,LAMA3,COL6A3,FGFR4,LAMB1,COL4A1,THBS1,PRLR,TNC,ITGB8,FGF7,PDGFRA,COL4A2,ITGB3,NR4A1,IL6,FN1,COL6A1,IBSP,AREG,EREG**
Malaria	0.003	11/45	TNF,IL1B,VCAM1,ITGB2,SELP,THBS1,IL18,CCL2,ICAM1,IL6,CCL12
NF-kappa B signaling pathway	0.008	15/93	TNF,CXCL1,IL1B,VCAM1,PTGS2,CD14,CCL4,PLAU,LBP,BCL2A1C,CXCL12,BCL2A1D,ICAM1,CXCL2,TNFSF13B

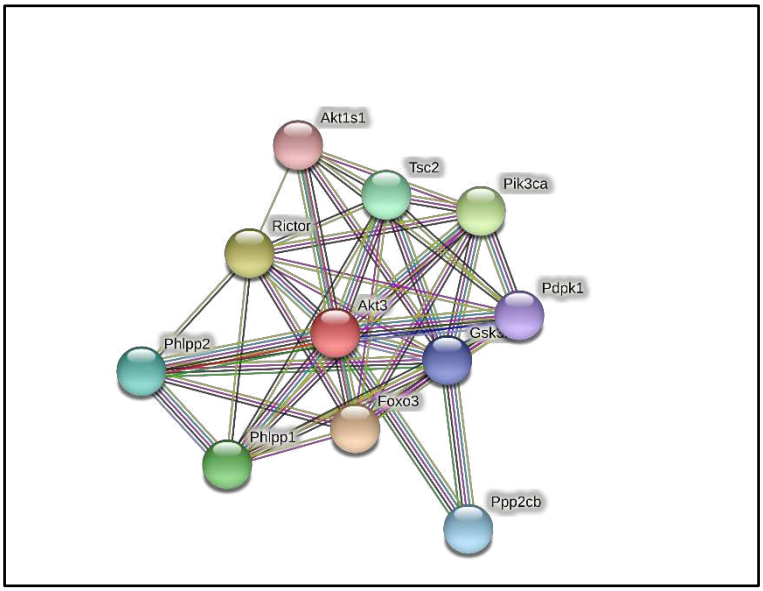


Figure 2. Strong interactions of differentially expressed genes in the pathway. The average local clustering coefficient was 0.9 and the protein-protein interaction enrichment p-value was $6X10^{-12}$ which demonstrated significant interactions among proteins and biological connections.

Results

Detection of infection and histological examination

The detection of the oocyst shedding was performed by qPCR confirming the infection in all inoculated animals. After histological examination of the ileo-caecal region, the presence of High Grade Intraepithelial Neoplasia (HGIEN) was detected in *C. parvum* infected animals at 60 days post-infection. The presence of the parasite in different stages were also identified (Figure 3).

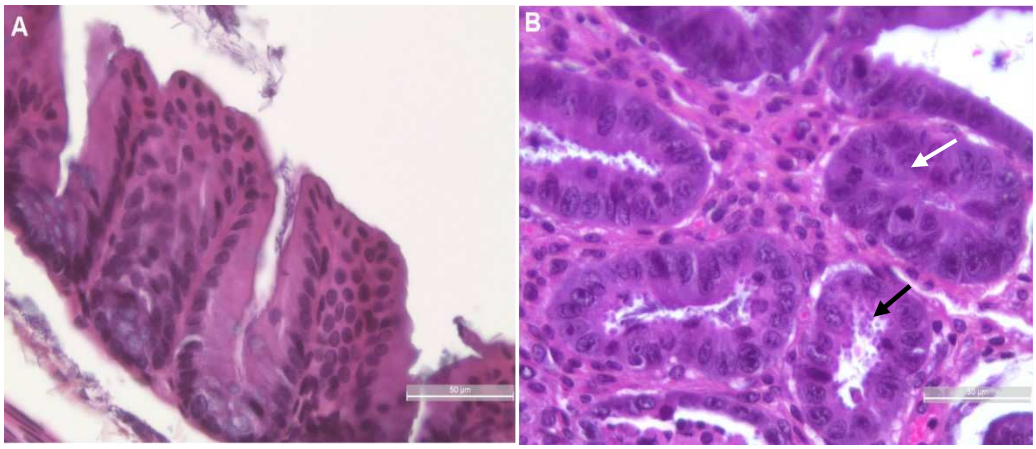


Figure 3. Histological examination of *C. parvum* infected (A) and non-infected (B) ileo-cecal region at day 60 PI. The presence of high grade intraepithelial neoplasia (HGIEN) detected in *C. parvum* infected tissue. The parasites are indicated with black arrow. A zone of HGIEN is shown (white arrow).

Results

Immunofluorescence assays in *C. parvum* infected SCID mice

Analysis of p85α, pAKT and AKT labelling:

Immunofluorescence studies revealed that there was a significant increase in the signal intensity corresponding to the expression of p85α in the ileo-cecal region of SCID mice infected with *C. parvum* at 60 days post-infection compared to the uninfected mice (Figure 4), especially in areas with cellular atypia and merged glandular region corresponding to some of typical criteria of intramucosal adenocarcinoma. The presence of the parasite at the apical region of the epithelial cells was confirmed by DAPI.

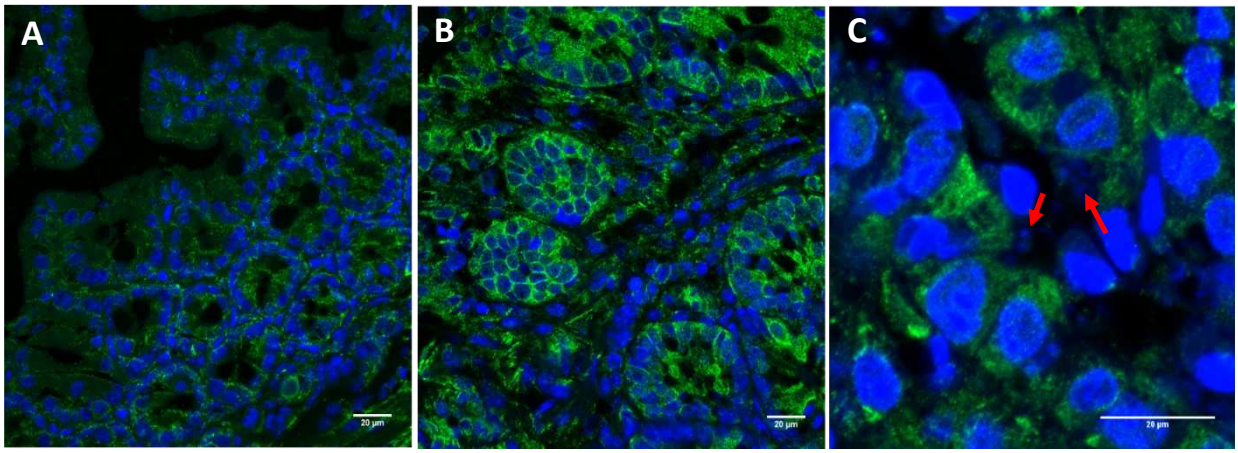


Figure 4. Immunofluorescence analysis of ileo-caecal regions of SCID mice immune-stained with a rabbit polyclonal anti- p85α antibody and an Alexa-Fluor-488-coupled secondary antibody. (A) Ileo-caecal region of an uninfected SCID mouse showing a low cytoplasmic labeling of p85α. (B and C) Ileo-caecal region of a *C. parvum* IOWA infected SCID mouse showed an increase in p85-α labeling at 60 days post-infection. Nuclear staining was done with DAPI. Parasites are shown (red arrows). Scale bar, 20 µm. The difference in the fluorescence intensity signals between infected *vs* non-infected tissue was statistically significant $p<0.05$.

Results

Concerning AKT, as a result of our immunofluorescence study we found that its endogenous cytoplasmic localization was not significantly upregulated in intestinal epithelial cells of the ileo-cecal region of *C. parvum* infected SCID mice compared to the non-infected mice (Figure 5).

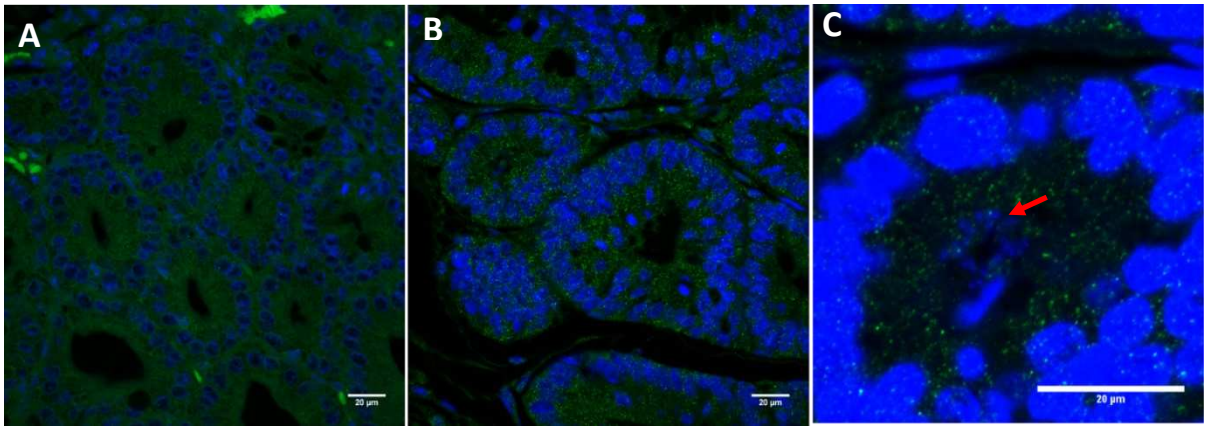


Figure 5. Immunofluorescence analysis of AKT in the ileo-caecal region of SCID mice – tissue sections were immune-stained with rabbit monoclonal anti-AKT antibody and an Alexa-Fluor-488-coupled secondary antibody. (A) Ileo-caecal region of an uninfected SCID mouse showing cytoplasmic labeling of AKT. (B and C) Ileo-caecal region of a *C. parvum* IOWA infected SCID mouse AKT at day 60 PI. Nuclear staining was done with DAPI. Parasites are shown (red arrows). Scale bar, 20 µm. The difference in the fluorescence intensity signals between infected *vs* non-infected tissue was not observed to be statistically significant.

However, we observed an increase in the labeling of phosphorylated AKT (Ser 473) (pAKT) in the ileo-cecal region of SCID mice infected with *C. parvum* at 60 days PI compared to the control non-infected mice (Figure 6). The localization of pAKT was cytoplasmic. The presence of parasites was confirmed by the DAPI staining. The staining in the chorion can be attributed to unspecific binding considering that similar intensity of the staining was observed in negative control slides (only secondary antibody staining).

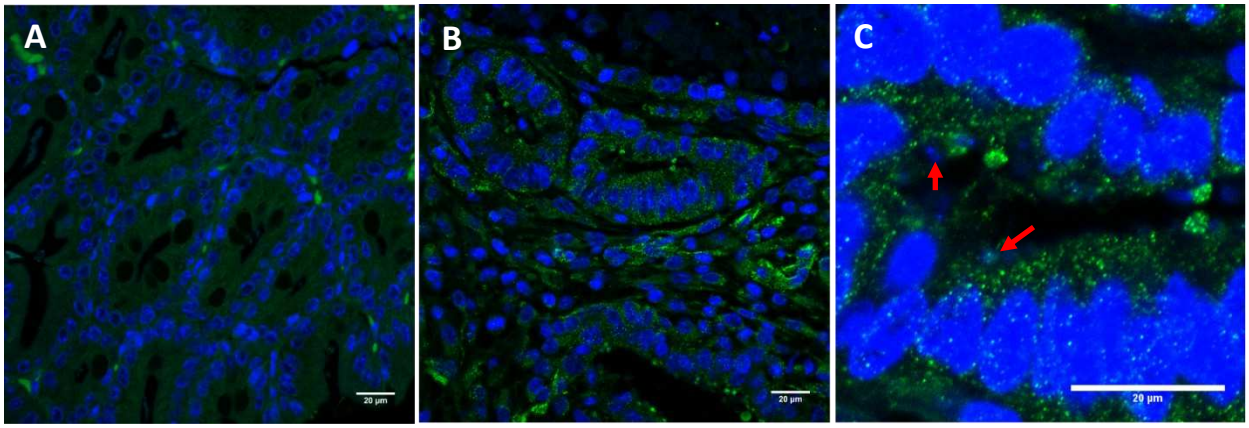


Figure 6. Immunofluorescence analysis of pAKT in the ileo-cecal region of SCID mice. – tissue sections were immune-stained with rabbit monoclonal anti-pAKT antibody and an Alexa-Fluor-488-coupled secondary antibody. (A) Ileo-cecal region of an uninfected SCID mouse showing a low cytoplasmic labeling of pAKT. (B and C) Ileo-cecal region of a *C. parvum* IOWA infected SCID mouse showing an increase in the labeling of pAKT. Nuclear staining was done with DAPI. Parasites are shown (red arrows). Scale bar, 20 µm. The difference in the fluorescence intensity signals between infected vs non-infected tissue was observed statistically significant $p<0.05$.

Results

Western blotting assays in *C. parvum* infected SCID mice

Analysis of p85α, pAKT and AKT expression:

To confirm the immunofluorescence results and the preliminary data described in the introduction, we performed western blotting analysis on the total protein extracted from ileo-caecal area of non-infected and infected SCID mice after 60 days PI. As a result, in concordance with immunofluorescence results, we observed an increase in the expression of the p-AKT and p-85α molecules in *C. parvum* infected tissue. Moreover, to confirm the activation of this subunit of PI3K, p85α was immunoprecipitated and using an anti-phosphotyrosine antibody its phosphorylated form was detected. We observed an increase tyrosine-phosphorylated p85α in *C. parvum* infected tissue (Figure 7).

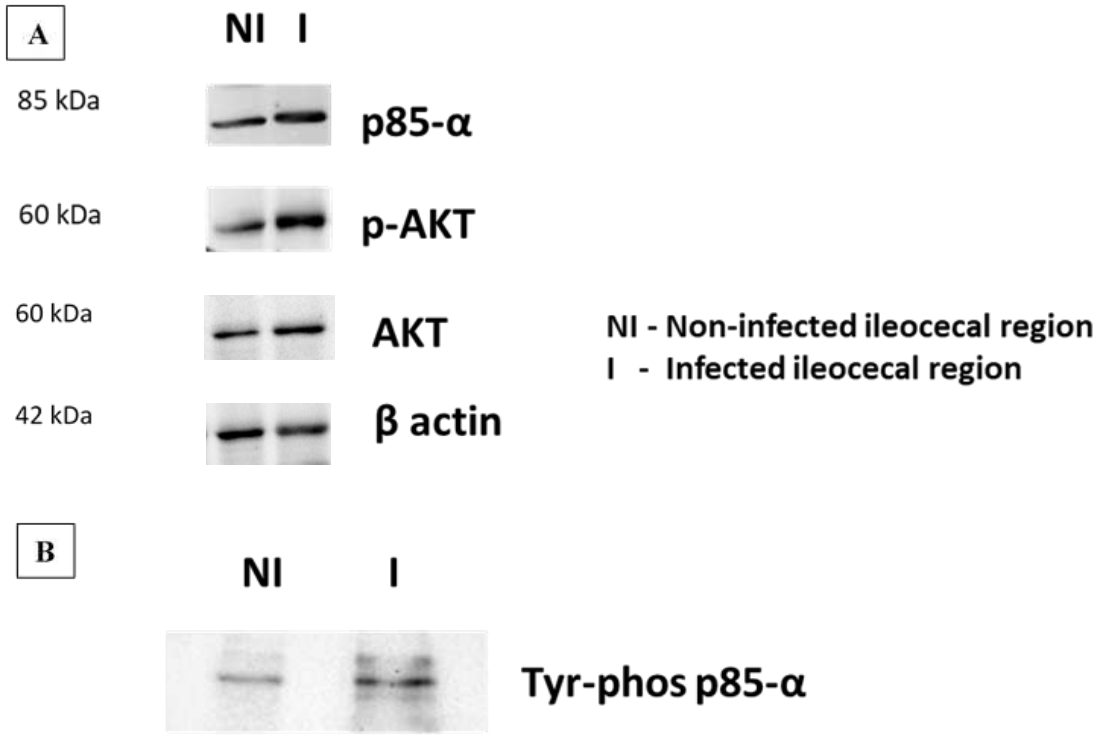


Figure 7. Western blotting analysis of PI3K/AKT pathway from caecum tissue of C. parvum uninfected and infected SCID mice.(A) Total cellular protein extract obtained from the ileo-caecal region was evaluated for the endogenous expression of p85α, p-AKT and AKT from non-infected and infected animals at 60 days PI. (B) Immunoprecipitation of p85α from non-infected and *C. parvum* infected caecum tissue at 60 days PI. Its phosphorylated status was detected using an anti-phosphotyrosine antibody. β-actin was used as a protein loading control. Protein concentration/well,10 µg/µl PI.

Discussion

The purpose of the study was to shed new insights on the mechanisms by which *C. parvum* induces the development of digestive neoplasia, and in particular to determine the role of the PI3K/AKT signaling pathway in this oncogenic process. PI3K/AKT signaling is one of the most extensively explored pathways in cancer pathogenesis and many drugs targeting this pathway are under development. Concerning colorectal cancer, the activation of PI3K/AKT is reported in about 40% of the malignant tumors (12). So, the question was to elucidate if the PI3K/AKT pathway was involved in the carcinogenic process induced by *C. parvum* in a rodent model. Indeed, other oncogenic pathogens such as Human papillomaviruses (HPV) increase their replication by altering cell signaling pathways to control transcription, regulate the cell cycle, inhibit apoptosis, evade host defense, and alter cellular metabolism (13). Particularly, it has been shown that activation of PI3K/AKT signaling occurs at different steps during the virus life cycle including (a) invasion, (b) release of proteins after virus delivery into the cell, (c) replication, and (d) virus latency and reactivation (13).

Firstly, the pathway enrichment analysis and more precisely, the KEEG and String analyzes revealed a link between the PI3K/AKT pathways and the extracellular environment, where the focal adhesion and the ECM (extracellular matrix) pathways seems to be upstream regulators of the PI3K/AKT. This observation is interesting given that some of the constituents of focal adhesions participate in the structural link between receptors located at the membrane and the actin cytoskeleton, whereas others are signaling molecules, including different protein kinases and phosphatases. These signaling events lead to reorganization of the actin cytoskeleton; and as a consequence changes in cell shape and motility, and gene expression can occur (14). Interestingly, the pro-oncogene tyrosine-protein kinase Src (HCK) also seems to play a role in this process. Src has been described as having a critical role in colorectal cancer which further activates downstream target signaling pathways, such as AKT, MAPK/ERK and FAK, and promotes Epithelial Mesenchymal Transition by downregulating E-cadherin and inducing β-catenin-dependent malignant processes (15).

Moreover, the immunofluorescence experiment helped to infer that overexpression of p85α might play a role in this oncogenic process. Western-blot analysis, confirmed that p85α was phosphorylated on the tyrosine residues, it is well known that this could be done by receptor tyrosine kinases (RTKs), to perform downstream signaling once activated (16). Hence, it could

be suggested that in *C. parvum* infected intestinal epithelial cells phosphorylated p85α, results in activation of PI3K which might be involved in phosphorylation of AKT.

In addition, this biochemical and cellular analysis allows highlighting hyperactivation of AKT which is known to be a hallmark of a wide variety of different human cancers such as ovarian (17), breast (18) and pancreatic (19). Moreover, cytoplasmic localization of pAKT is consistent with the results observed in sporadic colon carcinogenesis (20).

Indeed, AKT is considered as a critical signaling node in mammalian cells as a mediator of the majority of PI3K's downstream effects (17). Consistently, AKT3, one of the isoforms of AKT, was upregulated at day 93 PI as shown in the microarray study on ileo-caecal tissues of *C. parvum* infected SCID mice (10). We did not find upregulation of AKT in the immunofluorescence study, this probably because a pan AKT antibody was used.

Once phosphorylated, AKT localizes to subcellular compartments where it phosphorylates substrates that are related to different functions, such as cell growth, survival, apoptosis, and cell migration. However, the major role of AKT is associated with cell growth. Concerning its pro-migratory function, it has been reported that the alpha6beta4 integrin, a tumor-associated antigen, promotes cell migration and invasion in breast and colon cancer by activating PI3K/AKT signaling. Additionally, AKT can stimulate secretion of matrix metalloproteases that are required for degradation of the extra-cellular matrix (ECM). AKT signaling can also increases activation of several small GTPases leading to remodeling of the actin cytoskeleton and enhanced cell motility. Expression of AKT has also been associated with epithelial-mesenchymal transition (EMT), a process intimately associated with tumor progression to invasive and metastatic carcinoma (21).

In addition, glycogen synthase kinase 3 (GSK3) can be inhibited via AKT mediated phosphorylation, leading to ubiquitylation and proteasomal degradation of its targets involved in cellular metabolism (20). Interestingly, GSK3β may mediate an interaction and crosstalk between the PI3K/AKT and the WNT signaling pathways. This is consistent with the results of the String analysis and with the fact that the WNT signaling pathway seems to be implicated in the induction of ileo-caecal adenocarcinoma by *C. parvum* based on the alterations found in the expression of Apc and β-catenin in a rodent model of cryptosporidiosis (22).

Constitutively active AKT can also stimulate IKK activity. The IKK complex then phosphorylates both the IκB protein and the p65/RelA subunit, inducing enhanced activation of the NFκB transcription factor (23). NFκB is known to induce anti-apoptotic mechanisms

during *Cryptosporidium* infection to protect infected cells from death facilitating in this way parasite survival and multiplication (24).

Moreover, the PI3K/AKT can downregulate H3K27 and H3K4 methylations and this is consistent with results found recently by Sawant et al (article submitted to the journal "Virulence" and is under revision). This has been already described in gastric cancer, in association with an activation of the EMT (25).

In conclusion, the results obtained in this study showcase a potential role of PI3K/AKT signaling pathway in *C. parvum* induced carcinogenesis but further studies have to be done to confirm and better understand this mechanism. Even though activation of AKT might be governed by PI3K, some other different signaling pathways may regulate its expression. To explore this hypothesis, it would be interesting to use PI3K inhibitors such as wortammin or LY 294002 and to check for the endogenous expression of AKT in *C. parvum* infected mice. If the inhibition of PI3K downregulates the expression of AKT and in turn tumorigenicity is reduced, it could be inferred that AKT indeed acts as an oncogene via the PI3K signaling. It would be also important to define which isoform of AKT is responsible for this expression.

Acknowledgements

This study was supported by the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Institut Pasteur of Lille, the University of Lille and the Centre Hospitalier Regional Universitaire de Lille (CHU). M.S. was supported by a PhD fellowship from the University of Lille.

Author Contributions

Conceptualization, M.S., E.V. S.BV. and G.C.; funding acquisition, E.V.; investigation, M.S., C.C., E.D., L.L., S.BV..; project administration, E.V., S.BV, G.C.; supervision, S.BV. and G.C.; writing—original draft, M.S., S.BV. and G.C.

References

1. Certad, G. *et al*. *Cryptosporidium parvum*, a potential cause of colic adenocarcinoma. *Infect Agent Cancer*. **2**, 22 (2007).
2. Benamrouz, S. *et al.* Cryptosporidium parvum-induced ileo-caecal adenocarcinoma and Wnt signaling in a mouse model. *Dis. Model. Mech.* **7**, 693–700 (2014).
3. Tolias, K. F., Cantley, L. C. & Carpenter, C. L. Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* **270**, 17656–17659 (1995).

4. Koveitypour, Z. *et al.* Signaling pathways involved in colorectal cancer progression. *Cell Biosci.* **9**, 1–14 (2019).
5. Sever, R. & Brugge, J. S. Signal Transduction in Cancer. *Cold Spring Harb. Perspect. Med.* **5**, (2015).
6. Mele, R., Gomez Morales, M. A., Tosini, F. & Pozio, E. *Cryptosporidium parvum* at different developmental stages modulates host cell apoptosis in vitro. *Infect. Immun.* **72**, 6061–7 (2004).
7. Heussler, V.T. & Küenzi, P., Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. *Int J Parasitol*. 31, 1166-76 (2001).
8. Chen, X. M. *et al.* Phosphatidylinositol 3-kinase and frabin mediate *Cryptosporidium parvum* cellular invasion via activation of Cdc42. *J. Biol. Chem.* **279**, 31671–31678 (2004).
9. Vivanco, I. & Sawyers, C. L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489–501 (2002).
10. Sawant, M. *et al.* Persistent *Cryptosporidium parvum* Infection Leads to the Development of the Tumor Microenvironment in an Experimental Mouse Model: Results of a Microarray Approach. *Microorganisms* **9**, (2021).
11. Benamrouz, S. *et al. Cryptosporidium parvum* Infection in SCID Mice Infected with Only One Oocyst: qPCR Assessment of Parasite Replication in Tissues and Development of Digestive Cancer. *PLoS One* **7**, e51232 (2012).
12. Danielsen, S. A. *et al.* Portrait of the PI3K/AKT pathway in colorectal cancer ☆. (2014). doi:10.1016/j.bbcan.2014.09.008
13. Zhang, L., Wu, J., Ling, M. T., Zhao, L. & Zhao, K. N. The role of the PI3K/AKT/mTOR signaling pathway in human cancers induced by infection with human papillomaviruses. *Mol. Cancer* **14**, (2015).
14. Hofman, P. & Vouret-Craviari, V. Microbes-induced EMT at the crossroad of inflammation and cancer. *Gut Microbes* **3**, 176–185 (2012).
15. Jin, W. Regulation of Src Family Kinases during Colorectal Cancer Development and Its Clinical Implications. *Cancers (Basel).* **12**, (2020).
16. Yang, J. *et al.* Targeting PI3K in cancer: mechanisms and advances in clinical trials. *Mol. Cancer 2019 181* **18**, 1–28 (2019).
17. Zeng, Q. Y. *et al.* Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/AKT pathway in human ovarian cancer.

Oncogene **19**, 2324–2330 (2000).

18. Bellacosa, A. *et al.* Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. cancer* **64**, 280–285 (1995).
19. Altomare, D. A. *et al.* Frequent activation of AKT2 kinase in human pancreatic carcinomas. *J. Cell. Biochem.* **87**, 470–476 (2002).
20. Anderson, E. C. & Wong, M. H. Caught in the AKT: regulation of Wnt signaling in the intestine. *Gastroenterology* **139**, 718–722 (2010).
21. Duan, H., Qu, L. & Shou, C. Activation of EGFR-PI3K/AKT signaling is required for Mycoplasma hyorhinis-promoted gastric cancer cell migration. *Cancer Cell Int.* **14**, (2014).
22. Benamrouz, S. *et al.* Cryptosporidium parvum-induced ileo-caecal adenocarcinoma and Wnt signaling in a mouse model. *Dis. Model. Mech.* **7**, 693–700 (2014).
23. Bai, D., Ueno, L. & Vogt, P. K. AKT-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and AKT. *Int. J. cancer* **125**, 2863–2870 (2009).
24. Chen, X.-M. *et al. Cryptosporidium parvum* activates nuclear factor kappaB in biliary epithelia preventing epithelial cell apoptosis. *Gastroenterology* **120**, 1774–83 (2001).
25. Liu, X. *et al.* AURKA induces EMT by regulating histone modification through Wnt/β-catenin and PI3K/AKT signaling pathway in gastric cancer. *Oncotarget* **7**, 33152–33164 (2016).

Discussion

4. Discussion

Microbial pathogens employ numerous strategies to target cellular processes during their complex interactions with their hosts. Identifying pathogen-encoded proteins that manipulate host functions to promote infection and evade cellular responses can provide fundamental insights into host cellular processes. Furthermore, tumorigenic pathogens often target epigenetic mechanisms to hijack cellular host functions. Most studies of infection-related cancers have focused on viruses (e.g. human papillomavirus, hepatitis B and C, Epstein-Barr virus...) or bacteria (e.g. *Helicobacter pylori*).

The role of intracellular parasites in human cancer has been largely ignored. Several eukaryotic parasites are among the suspected causes of infectious cancer and experimental data have confirmed that intracellular eukaryotic parasites can interfere with the host cell machinery to hijack intracellular processes. For this reason, during this thesis project, we focused on the study of the interactions between *Cryptosporidium* and its host to understand pathophysiological aspects of the infection leading to parasite-initiated cancer.

4.1. Importance of histone lysine methylations in *C. parvum*

Cryptosporidium species have a very compact genome (~9.2 Mb) unlike its apicomplexan homologs such as *Toxoplasma* (~63 Mb) and *Plasmodium* (~22.8) Mb (Abrahamsen MS et al. 2004; Gardner MJ et al. 2002; Kissinger JC et al. 2003). Comparative analysis of genomic diversity between Apicomplexa (*Plasmodium* and *Cryptosporidium*) and other eukaryotes revealed that the two parasites have considerable qualitative differences in conserved protein architectures associated with chromatin dynamics and transcription (Templeton TJ et al. 2004). The study predicted about 340 and 800 specific transcription factors in *Cryptosporidium* and *Plasmodium* respectively. However, it was noted that these numbers were far fewer in comparison to other eukaryotes, suggesting that there exist major differences in the mechanisms of Apicomplexan gene regulation. The scarcity of transcription factors in *Cryptosporidium* and other Apicomplexa suggests that the regulation of transcription might be dependent on other systems such as chromatin-remodeling apparatus (Hakimi MA and Dietsch KW 2007; Templeton TJ et al. 2004). Moreover, *Cryptosporidium* has been predicted to have 14 chromatin-remodeling SNF2/SWI2 ATPases, whereas *Plasmodium* has just 11. In addition, several chromatin proteins have been predicted with unique domain architectures which are unseen in other eukaryotes. Some unique architectures exclusive to *Cryptosporidium*

chromatin protein were predicted for proteins such as cgd4_1590, an ISWI-related SNF2/SWI2 ATPase accompanied at N-terminal with two chromodomains and one bromodomain (Templeton TJ et al. 2004). In particular, the fact that Apicomplexan nuclear proteins are capable of having novel architectures highlights the significance of this mechanism unique to this lineage. On the other hand, *Toxoplasma* displays a distinctly higher number of Apicomplexan Apetala 2 (ApiAP2) transcription factors, in comparison to all other Apicomplexans (Iyer LM et al. 2008). Interestingly, *Cryptosporidium* is less dependent on the ApiAP2 regulators potentially because it relies on E2F/DP1 transcription factors (Oberstaller J et al. 2014). Thus, it can be speculated that *Cryptosporidium* is heavily reliant on its epigenetic mechanisms. In Apicomplexans, the chromatin-remodeling proteins have been speculated to co-ordinate with specific DNA-binding proteins detected in their genome to carry out gene regulation (Templeton TJ et al. 2004). Putative *C. parvum* DNA methyltransferases (DNMT2) orthologs showed full-length alignment with other eukaryotes and sequence similarity of conserved residues of the catalytically active site (Templeton TJ et al. 2004). However, mass spectrometry analysis performed to investigate the participation of these enzymes in an epigenetic control mechanism related to transcriptional cascading revealed that *Cryptosporidium* lacked detectable methylated DNA (Gissot M et al. 2008). Strikingly, there is a prominent inter-dependency of chromatin-remodeling proteins such as SNF2/SWI2 ATPases and histone methylases (Iyer LM et al. 2008). The presence of 14 SNF2/SWI2 ATPases detected in *Cryptosporidium*, strengthens the speculation that histone methylation is one of the prominent epigenetic mechanisms occurring in the parasite.

4.2. Chromatin vs other protein lysine methyltransferases of *C. parvum*

Out of the many domains found in the chromatin proteins of the *Cryptosporidium* genome, SET domain and cysteine clusters associated with SET domains were also detected (Templeton TJ et al. 2004). The chromatin proteins which are known to contain the SET domain are known as histone methylases (Iyer LM et al. 2008). All SET domain proteins catalyze the transfer of the methyl group on a lysine residue of its substrate (Cheng X et al. 2005). Hence, chromatin proteins that bring about methylation of histones are termed as HKMTs. Thus, the speculated prominent epigenetic mechanisms of histone methylations in *Cryptosporidium* are predicted to be carried out by the SET domain containing HKMTs.

The most widely characterized lysine residues for HKMTs to catalyze the methyl group transfer are the ones located on the N-terminal tails of histones (such as at K-4, K-9, K-27, K-36 of

histone H3, and K-20 of H4) that protrude out from the nucleosome (Sims RJ III et al. 2003). Different families of HKMTs are known to methylate different specific Lys residues (Sims RJ III et al. 2003).

Based on these observations, the first objective of my thesis was to study the role of epigenetics in the interaction between *Cryptosporidium* and its host. Considering that epigenetics has an important role in transcription regulation, we carried out a study to investigate histone lysine methylation, as a dynamic epigenetic modification during the life cycle of the parasite. CpSET1, CpSET2, and CpSET8 were characterized as potential HKMTs of *C. parvum*. The presence of the plant homeodomain (PHD) domain and bromodomain assisted in identifying these proteins to be chromatin proteins (Templeton TJ et al. 2004). Moreover, proteins containing the PHD domain have been identified for their histone binding capabilities (Jain K et al. 2020). The conservation of key active site residues responsible for lysine substrate specificities classifies the HKMTs into different families (Dillon SC et al. 2005). The recognition of the target lysine residue by a specific HKMT is achieved through a variety of interactions between specific amino acid residues of HKMT and the surrounding histone protein sequences (Zhang X et al. 2003). The phylogenetic analysis clustered CpSET1, CpSET2 and CpSET8 into different HKMT families indicating that the specific amino acid residues of CpSETs required to interact with histones to target their specific lysine residue are well-conserved. The transfer of the methyl group on the target lysine residue is a result of the most conserved residues of the SET domain. The structural organization of these residues gives rise to a pseudoknot structure to form an active site (Dillon SC et al. 2005). Homology modeling analysis of SET domains of CpSET1, CpSET2, and CpSET8 revealed that *C. parvum* HKMTs structurally conserve this pseudoknot architecture, and predicted them to be structurally active.

The tyrosine to phenylalanine residue switch (F/Y switch) of certain conserved residues present within this pseudoknot structure determines whether the enzyme would mono-, di, or tri methylate the histone (Dillon SC et al. 2005). However, when there is the existence of two tyrosine residues the methyltransferase activity is restricted to mono-methylation (Nishioka K et al. 2002). This was the case identified for CpSET1 which conserved the (Tyr 2129 Tyr 2217) required for the transfer of methyl group (Article 1; Figure 3A). Interestingly, superimposition analysis allowed us to further identify CpSET1 to conserve the residues (Cys 2156 to Phe 2158) which allows free movement of the lysine side chain (Article 1; Figure 3A). This is a characteristic feature of the MLL1 subfamily of HKMTs which mono-, di- and tri-methylates

H3 K4 (Southhall SM et al. 2009). Thus, CpSET1 can be predicted to mono, di or tri-methylate H3K4. Interestingly, structural alignment revealed that in CpSET8 tyrosine to phenylalanine residue switch takes place (Tyr 445 and Phe 537) (Article 1; Figure 3C). Thus, suggesting that CpSET8 is capable of adding multiple methyl group to its target lysine(Article 1; Figure 4). Similar to its other Apicomplexa orthologs such as *T. gondii* and *P. falciparum* (Sautel CF et al. 2007). Whereas, for CpSET2 the conservation of an arginine residue (Arg 548) determined it to tri-methylate H3K36 (Article 1, Figure 3B). Mutating arginine residue (Arg1625Cys) present in the SET domain results in an enzymatically inactive SETD2 failing to tri-methylate H3K36 (Hacker KE et al. 2016). CpSET2 showed 43 % structural identity to SETD2.

These *Cryptosporidium* HKMTs were functionally characterized by performing immunofluorescence assays using anti-methylation antibodies during the life cycle of the parasite. Consistent labeling of the H3K4Me3 methylation mark accompanied by high expression of the *CpSET1* gene during all the stages of the parasite was observed (Article 1, Figure 5B). H3K4Me3 has been shown to mark the promoter of actively transcribed genes in different Apicomplexan parasites such as *T. gondii* (Gissot M and KIM K 2008), *T. annulata* (Cheeseman, K et al. 2021) and *P. falciparum* (Lopez-Rubio JJ et al. 2007). Thus, CpSET1 can be predicted to be responsible for H3K4 methylation of a stable mark in the expression of actively transcribing genes during each stage of the parasite (Article 1; Figure 3A, 5B). H3K36Me3 methylation mark appeared mainly during the sexual stages of the parasite (Article 1; Figure 5B). Whereas the *CpSET2* gene was highly expressed during the asexual stage of the parasite (Article 1; Figure 5A). Thus, CpSET2 can be speculated to be more dynamic in its function (Article 1; supplementary figure 2A, B). However, whether it has a role in gene activation or gene repression is debatable (Figure 6). H3K36Me3 has been reported to be a mark of gene activation in higher eukaryotes (Sims RJ III et al. 2003). But methylation of H3K36 has been linked to repression of *var* genes in *Plasmodium* parasites (Jiang L et al. 2013). In contrast, the *Theileria* genome did not show any regions of strikingly high H3K36Me3 enrichment (Cheeseman, K et al. 2021). CpSET8 is also predicted to be dynamic with the H4K20Me3 methylation mark detected majorly during sexual compared to asexual stages (Article 1; supplementary figure 2A,B). CpSET8 function can be predicted to be cell cycle-dependent similar to its Apicomplexan orthologs, *T. gondii, and P. falciparum* (Sautel CF et al. 2007). H4K20 methylation is regarded as a gene repression mark present at the heterochromatin region in Apicomplexa as well as higher eukaryotes (Sautel CF et al. 2007;

Sims RJ III et al. 2003). Thus, the same is expected for CpSET8 regulated H4K20 methylation (Figure 6).

In parallel, non-histone protein methylation has also been identified as an important cellular function in higher eukaryotes. Similar to histones, a non-histone protein can undergo methylations at Lys and Arg residues. HKMTs such as G9a, can mono- or di-methylate a minimum of 17 non-histone proteins (Biggar K. K and Shawn S.-C. Li 2015). However, HKMTs are chromatin-enzymes that are concentrated in the nucleus. Thus, most of their non-histone protein targets are transcription factors and other nuclear proteins such as p53 and NFκβ which regulate cellular functions (Egorova KS et al. 2010). For instance, a chloroplast protein (Rubisco, for ribulose- 1,5-bisphosphate carboxylase/oxygenase) (Houtz RL et al. 1992), a mitochondria protein (cytochrome C) (Pollock WB et al. 1998), growth factor receptor (vascular endothelial growth factor receptor 1) (Kunizaki M et al. 2007) and ribosomal proteins (Porras-Yakushi TR et al. 2007) have been described as non-nuclear lysine methyltransferases. In our research project, phylogenetic analysis revealed that CpAKMT and its homologues from other Apicomplexa clustered together and represented a sister-group of HsSMYD3, as described in previous evolutionary studies including *Plasmodium* (Cui L et al. 2008) and *Toxoplasma* KMTs (Sivagurunathan S et al. 2013). The lack of an MNYD domain which is a peculiarity of the SMYD family of HKMTs and the non-nuclear localization of AKMT of *T. gondii* (Heaslip AT et al. 2011) has regarded these proteins to have non-nuclear functions. However, they have been shown to perform methyltransferase activity *in vitro* (Sivagurunathan S et al. 2013). Thus, this distinct clade of KMTs can be characterized as non-nuclear lysine methyltransferases specific to the phylum Apicomplexa. The possibility that CpAKMT may be able to methylate extra-nuclear histones of *C. parvum* cannot be excluded and further studies are needed to clarify this aspect.

CpKMTox is another example of an unusual KMT identified in *Cryptosporidium* which could form a distinct clade of Apicomplexa enzymes. In *T. gondii*, KMTox has been shown to interact with typical 2-cys peroxiredoxin-1 (TgPrx1) and regulate the expression of genes involved in antioxidant defences and maintenance of cellular homeostasis (Sautel CF et al. 2009). The same function in yeast is brought about by transcription factors. In yeast, in response to peroxide stress, a transcription factor, Yap1 is retained in the nucleus to activate target genes by the formation of disulfide bonds induced by Gpx3, a peroxidase (Rodrigues-Pousada CA et al. 2004). Thus, this example shows the reliance of Apicomplexan parasites on epigenetic mechanisms, especially histone lysine methylations to regulate cellular functions.

Interestingly, in higher eukaryotes, until the discovery of histone demethylases, this post-translational modification was regarded to be stable (Trojer P and Reinberg D 2006). KDMs containing JmjC-domain were not identified in *Cryptosporidium*, highlighting that the mechanism for histone demethylation is not present in this parasite as reported in previous studies (Cui L et al. 2008; Rider SD Jr and Zhu G 2010). Thus, histone lysine methylations can be considered to be stable modifications in *Cryptosporidium*. However, the existence of an alternative mechanism cannot be denied.

4.3. *C. parvum* mediated survival strategies for the persistence of the infection

Different studies suggest that *C. parvum* has developed multiple strategies to evade the immune response to facilitate its multiplication after infection (Laurent F and Lacroix-Lamandé S 2017). Among these strategies different mechanisms have been proposed as follows: inhibition of antimicrobial peptides released by the infected cells through the reduction of CCL20 expression. CCL20 displays antimicrobial activity against *C. parvum*, but its expression is reduced during infection in the intestine of neonatal mice (Guesdon W et al. 2015), restoration of the tryptophan availability in the epithelial cells needed for parasite development via the modulation of STAT1 and IDO1 expression (Choudhry N et al. 2009), modulation of apoptosis (McCole DF et al. 2000), delivering of parasite transcripts into the nuclei of infected cells to suppress host cell gene transcription involved in cell proliferation and amino-acid transport via epigenetic mechanisms (Wang Y et al. 2017).

Interestingly, the regulation of epigenetic mechanisms of the host has been one of the strategies of several intracellular pathogens to survive (Villares M, Berthelet J, and Weitzman JB 2020). In the current research project, the study of the histone lysine methylations of the host allowed us to determine the downregulation of H3K4Me3 and H3K27Me3 marks as a consequence of *C. parvum* infection. *In vitro* analysis, revealed that these methylation marks were relatively high during the trophozoite stage of the parasite, however, they decreased predominantly during sexual stages (Article 1; Supplementary figure 4A). Consistently, downregulation of the H3K4Me3 mark has also been reported on the promoter of NF-κB-related pro-inflammatory genes in *Leishmania* infected macrophages as a survival strategy of the parasite (Lecoeur H et al. 2020). Unlike *C. parvum*, *T. gondii* infection induces upregulation of the H3K27Me3 mark. This upregulated level of methylation represses pro-inflammatory cytokines genes to allow the persistence of the infection (Braun L et al. 2019). Thus, regulation of histone lysine

methylation levels could be suggested as one of the immune escape strategies of *C. parvum* (Figure 6).

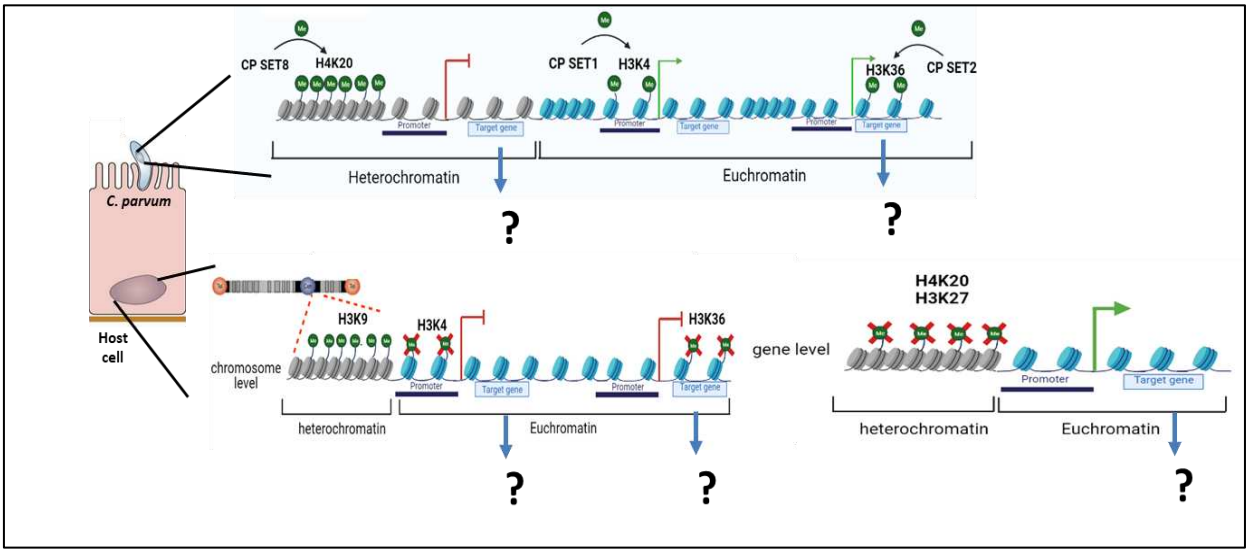


Figure 6. Schematic representation of histone lysine methyltransferase mediated epigenetic regulation during *C. parvum* infection. *C. parvum* CpSET1, 2, and 8 are predicted to methylate H3K4, H3K36, and H4K20. *C. parvum* is speculated to modulate host lysine methylations at chromosomal level (H3K9, H3K4, H3K27, and H4K20) and gene-level (H3K36). The red cross on the methyl group (green) indicates the downregulation of lysine methylation levels (H3K4, H3K36, H3K27, H4K20). Heterochromatin region : grey; Euchromatin region : blue. Red stop arrow : repression of gene expression. Green arrow : activation of gene expression. Image created using biorender.com.

In parallel, the study of signaling pathways involved in *C. parvum* infection and induced neoplasia by a microarray approach allowed us to identify that *C. parvum* was able to actively infect the host by resisting the upregulated expression of IFN-γ stimulated host immune response genes such as *GBPs* and *IIGP1*. Guanylate binding proteins (GBPs), *GBP2, GBP4, GBP6, GBP8,* and *GBP11*, a superfamily of large GTPases are induced by IFNγ as a host response to external pathogens (Currey N et al. 2019). Especially, *GBP2* and *IIGP1* have been identified to be recruited by the host at the host-parasite interface, to disrupt the parasitophorous vacuole in *T. gondii* (Foltz C et al. 2017; Martens S et al. 2005). Interestingly, *T. gondii* can evade this IFN-γ stimulated host immune response. Pseudokinase ROP54 secreted by the parasite can restrict the GBP2 loading onto the parasitophorous vacuole to evade GBP2-mediated immune response (Zhu W et al. 2019). In addition, Rhoptry protein kinase, ROP17, secreted during invasion can phosphorylate and eventually inactivate IIGP1 (Zhu W et al.

2019). Thus, *C. parvum* can also be speculated to resist the innate immune response to persist in the host (Figure 7) .

The inflammatory response generated as a result of resisting the upregulated expression of innate immune response genes has been speculated to downregulate the expression of anti-microbial peptides such as α-defensins (Figure 7). The molecular network analysis identified *TNFα* and *IGF1* regulated signaling pathways to downregulate the expression of α-defensins *DEFA1* and *DEFA4* respectively (Article 2; Figure 4). Thus, potentially identifying novel targets of *C. parvum* immune escape strategy.

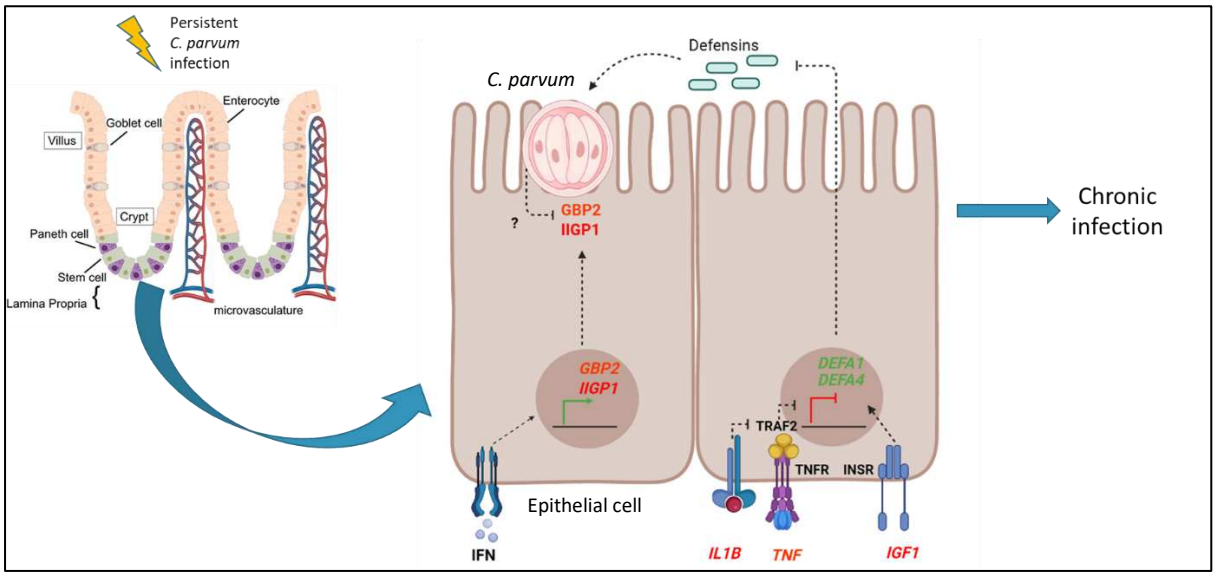


Figure 7. Hypothetical representation of potential mechanisms involved in the persistence of *C. parvum* infection in the intestine of the experimental mouse model. The representation is based on the molecular network (Article 4; Figure 4) at day 93 PI in intestinal epithelial cells. Upregulated expression of IFNγ-stimulated genes (*GBP2* and *IIGP1*) are resisted by *C. parvum* by an unknown mechanism (indicated in red). *IL1β*, *TNF,* and *IGF1* regulated signaling pathways are predicted to downregulate the gene expression of α-defensins (*DEFA1* and *DEFA4*) (indicated in green). Image created using biorender.com.

The ability of the parasite to evade the immunological response by resisting the upregulated expression of IFNγ-stimulated genes and downregulating the expression of key players such as α-defensins favor a persistent *C. parvum* multiplication and alteration of the balance towards cancer. Consistently, it is well known that oncogenic pathogens even if they are phylogenetically diversified, can sabotage signaling pathways to favor survival and transmission, and avoid the immune system (Ewald PW 2009).

4.4. *C. parvum* modulated mechanisms to induce digestive neoplasia

The study of the dynamic changes that occur in the host cell after *C. parvum* infection was of significant interest to understanding potential mechanisms involved in *C. parvum* induced digestive cancer.

Deregulation of epigenetic mechanisms especially histone lysine methylations has been widely studied in the process of carcinogenesis. In particular, deregulation of SET-domain containing proteins plays role in cancer (Schneider R et al. 2002). In this view, a parasite *T. annulata* has been shown to induce cellular transformation by upregulating the expression of MMP9, a molecule involved in EMT, through elevated levels of SMYD3 methyltransferases. However, it is currently unclear as to how the parasite induces SMYD3 expression whether it is through parasite secreted effector protein or host signaling pathways (Cheeseman, K et al. 2021).

Then, one of the strategies of my thesis project was to study the impact of the infection on the histone lysine methylations of the host. Strikingly, the modulation of different lysine methylation marks in *C. parvum* infected mice was described as follows: H3K9me3 was upregulated in the tissues of infected mice while H3K27Me3, H4K20Me3, H3K4Me3, and H3K36Me3 were downregulated. Moreover, *C. parvum* infection has already been shown to induce transcriptional suppression of host genes by promoter enrichment of H3K9me3 resulting in dysregulated transcription of host genes considered as key to intestinal epithelial homeostasis, including members of the LRP family of transcriptional regulators such as *LRP5*, and soluble carriers such as *SLC7A8* (Wang Y et al. 2017). Consistently, in our microarray study genes of both groups were downregulated such as *LRP12*, *Slc37a2,* and *Slca9*. Further studies are needed to elucidate this aspect.

In addition, H3K9Me3 and H3K4Me3 are considered to be opposing and mutually exclusive chromatin modifications (Zhang T, Cooper S, and Brockforff N 2015). Considering that H3K27Me3 and H4K20Me3 are markers of gene repression (Greer EL and Shi Y 2012), downregulation of these markers suggests that *C. parvum* could epigenetically manipulate the host epigenome to propagate the infection.

The microarray analysis gave us additional clues about the pathogenicity of the infection and the process of *C. parvum*-induced neoplasia. The overexpression of IFN-γ stimulated superfamily of GTPases (*IIGP1* and *GBPs)* is involved in the inflamed colon and restricted to the immediate proximity of the damaged epithelial barrier to assist propagation of inflammation assisted cancer development (Currey N et al. 2019). Moreover, attenuated

expression of α-defensins is responsible for compromising the host immunity favoring parasite multiplication, and altering the balance towards inflammation (Ramasundara M et al. 2009). Thus, the downregulation of α-defensins in transformed tissues as a result of *C. parvum* infection highlights the contribution of systemic inflammation to the parasite-induced carcinogenesis (Article 2; Figure 3A,4B).

Moreover, *AKT3*, one of the isoforms of *AKT* was observed to be upregulated within the dataset at day 93 PI (Article 2; Figure 5B). The oncogenic serine/threonine kinase AKT (also known as PKB), is a downstream effector of PI3K, shown to induce EMT (Grille SJ et al. 2003). Along with the molecular detection, preliminary results of immunofluorescence (Article 3, Figure 3-5) and western blotting analysis (Article 3, Figure 6), together with the gene enrichment analysis predicted PI3K/AKT signaling pathway (Article 3; Table 1, Figure 1) to be activated as a result of *C. parvum* induced tumorigenesis.

Interestingly, the PI3K/AKT signaling pathway known to be activated during *Cryptosporidium* infection (Forney JR et al. 1999) has been shown to downregulate H3K27 and H3K4 methylations with the activation of EMT in other types of cancer such as gastric cancers (Liu X et al. 2016) explaining in part the transformation process that takes place in *Cryptosporidium* infected epithelial cells. These results are consistent with our microarray study reporting that EMT takes place within the tumor microenvironment induced by *C. parvum* infection in a rodent model. Further studies such as chromatin immunoprecipitation sequencing have to be conducted to better elucidate this aspect.

We have discussed in the review "*Cryptosporidium* and colon cancer: cause or consequence" about hypothetical host-cell signaling pathways linking *Cryptosporidium* infection and oncogenesis. Different studies have shown that *C. parvum* induces cytoskeleton remodeling and actin reorganization through the implication of several intracellular signals, including phosphatidylinositol 3-kinase (PI3K). The results presented herein reinforce this speculation.

4.5. *C. parvum* infection results in an immunosuppressive tumor microenvironment

Molecular network analysis performed on the datasets revealed that inflammatory stimuli triggered by *IFNγ* and *TNF-α* via Signal transducer and activator of transcription 1 (*STAT1*) result in upregulation of *IDO1* in IECs (Article 2; Figure 6A). *IDO1* is an immunosuppressive cell signaling molecule. *IDO1* is responsible for catabolism of tryptophan within a tumor microenvironment. Unavailability of the tryptophan arrests T cell proliferation and induces

apoptosis which results in immune escape of the cancer cells (Nagaraj S. and Gabrilovich DI 2008). Inflammatory stimuli-mediated EMT has been shown to confer immunoregulatory properties to neoplastic epithelial cells by activation of *IDO1* and enhancing tumor immune escape (Ricciardi M et al. 2015). In parallel, *IDO1* expression in Paneth cells has been observed to be strictly regulated by *STAT1* and regarded as an immunosurveillance escape strategy of colorectal cancer (Pflügler S et al. 2020). Thus, validating our molecular network analysis.

Within a tumor microenvironment, high expression of *IDO1* is also indicative of the presence of a population of tumor-infiltrating immune cells such as myeloid-derived suppressor cells (MDSCs) which are known to have suppressive effect on adaptive immune responses (Nagaraj S. and Gabrilovich DI 2008). The presence of MDSCs in tumor microenvironment induced by *C. parvum* can also be predicted by the detection of upregulated expression of *ARG1*, another immune suppressive factor which depletes arginine in the microenvironment and hindsight protects the tumor from immune attack (Nagaraj S. and Gabrilovich DI 2008). Thus, giving the tumor microenvironment its immunosuppressive characteristic.

Upregulated expression of several growth factors (*IGF1,* fibroblast growth factor 7 (*FGF7*)), chemokine (C-X-C Motif Chemokine Ligand 12, (*CXCL12*)), proteases (matrix metalloproteinase 2 (*MMP2*)) and extracellular matrix (ECM) constituents such as secreted phosphoprotein 1 (*SSP1*) speculated the microenvironment to be enriched in cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) (Peddareddigari VG et al. 2010). Molecules such as *FGF* and *CXCL12* released from CAFs contribute to the maintenance of tumor growth and angiogenesis (Peddareddigari VG et al. 2010). Immune suppressive factors such as arginase1 (*ARG1*) and *SPP1* induce polarization of TAMs giving rise to tumor promoting M2 TAMs (Shurin MR 2018; Solinas G et al. 2009). Upregulated expression of *PTGS2*, can be predicted to increase prostaglandin production which in turn would assist the TAMs in inducing inflammation and promoting tumor growth (Eberhart CE et al. 1994; Solinas G et al. 2009). Identification of *STAT1* and *NF-κβ* as transcriptional regulators at the core of this tumor microenvironment network (Article 2; Figure 6B) highlights the role of chronic inflammation which assists in the propagation of cancer. This is similar to what happens in *H. pylori* infection that induces gastric cancer (Wang D and Dubois RN 2015).

Conclusions and Perspectives

5. Conclusions and Perspectives

The work carried out as part of this thesis accomplishes the main objective of contributing to the understanding of *Cryptosporidium*-host interactions. Results from this work allow us to confirm that the parasite can sabotage cellular pathways to favor a cellular environment propitious for parasite multiplication. Some of these pathways' deregulation can lead to cellular proliferation, inhibition of apoptosis, and immune evasion.

Particularly, the study of the epigenetic mechanism, histone lysine methylations, provided us for the first time with knowledge about a yet unknown cellular regulation that occurs in *C. parvum* to maintain its life cycle. In addition, observations about histone lysine methylations of the host together with the microarray results suggest that uncontrolled *C. parvum* infection is capable of hijacking and altering host cellular mechanisms leading to cancer development.

Specific conclusions are as follows:

- Protein sequence alignment and phylogenetic analysis identified five KMT as novel epigenetic regulators in *C. parvum*. CpSET1, CpSET2, and CpSET8 were identified to be HKMTs. Whereas, CpAKMT and CpKMTox were identified as KMTs exclusively found in the phylum Apicomplexa.
- 3-D homology modelling and superimposition analysis revealed CpSET1, CpSET2, and CpSET8 to be structurally active HKMTs in *C. parvum*.
- CpSET1, CpSET2, and CpSET8 were functionally predicted to methylate H3K4, H3K36, and H4K20 and regulate gene expression during *C. parvum* life cycle progression *in vitro*. Particularly, the methyltransferase activity of the SET domain of CpSET8 was showcased confirming the existence of functional HKMTs in *Cryptosporidium.*
- *C. parvum* was shown to manipulate the histone lysine methylations of the host by downregulating different lysine methylation marks either during the asexual or sexual developmental stages of the parasite. In particular, the downregulation of gene activation marks such as H3K36Me3 and gene repression marks such as H3K27Me3 represented the survival strategy of the parasite to persist in the infection and potentially have a role in the induction of digestive neoplasia (Figure 8).

- Modulation of the expression of IFNγ-stimulated genes and α-defensins can be considered as novel strategies of the parasite to evade the immune response and persist the infection (Figure 8).

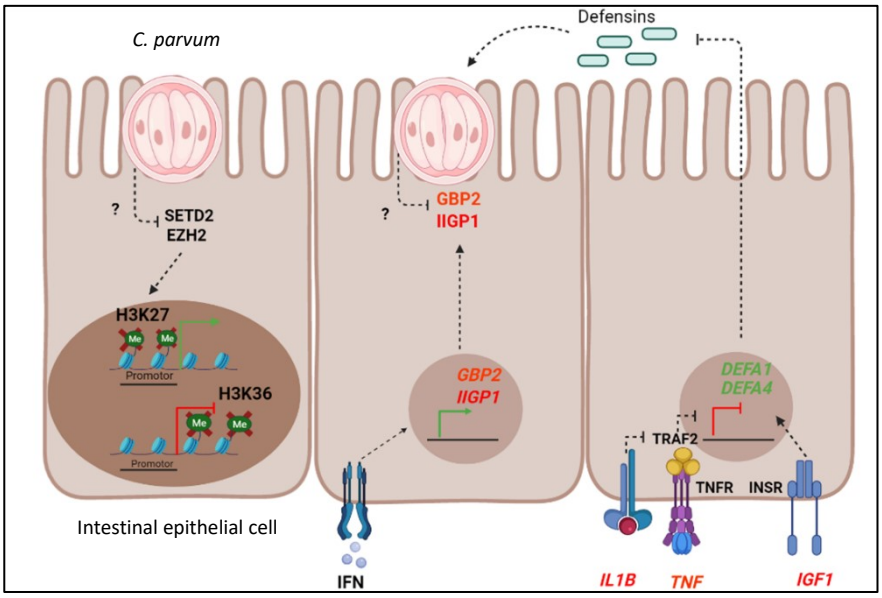


Figure 8. Schematic representation of epigenetic and transcriptomic modifications induced by *C. parvum* to persist the infection in the host cell. *C. parvum* is speculated to interfere with epigenetic regulators such as SETD2 and EZH2 of methylation marks such as H3K36 and H3K27 respectively. The interference leads to downregulation (red arrow) and upregulation (green arrow) of host genes favorable for the propagation of the parasite. In parallel, upregulated expression of IFNγ-stimulated genes (*GBP2* and *IIGP1*) (indicated in red text) are resisted by *C. parvum* by an unknown mechanism. *IL1β*, *TNF,* and *IGF1* regulated signaling pathways are predicted to downregulate the gene expression of α-defensins (*DEFA1* and *DEFA4*) (indicated in green text). Image created using biorender.com.

Conclusions and Perspectives

- *C. parvum*-induced neoplasia seems to be the result of global gene expression profile alterations. Inflammatory stimuli generated as a result of persistent infection assists in the propagation of epithelial to mesenchymal transition (EMT). In particular, the potential involvement of the PI3K/AKT signaling pathway at the intersection of *C. parvum* infection and onset of cancer was shown.

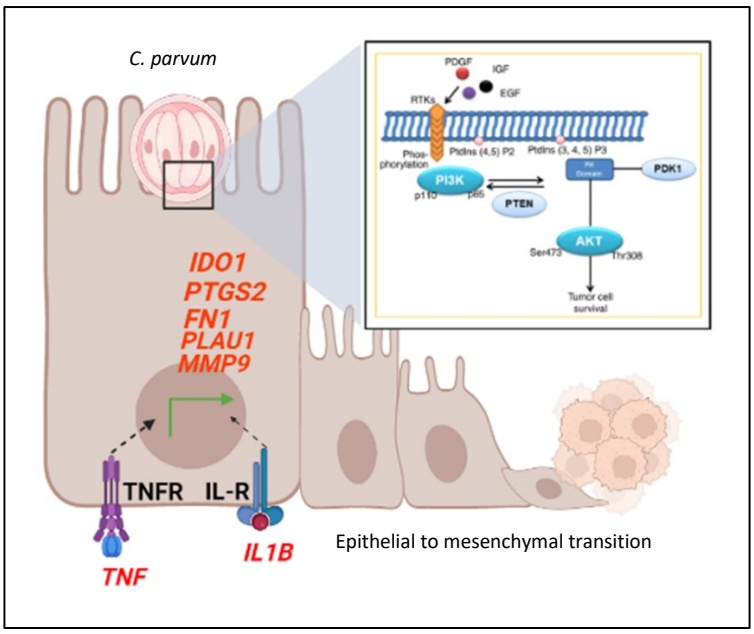


Figure 9. Schematic representation of inflammatory stimuli and PI3K/AKT pathway involved *C. parvum* induced carcinogenesis. As part of inflammatory stimuli, upregulated expression of pro-inflammatory cytokines such as *TNF* and *IL1β* regulated signaling pathways are predicted to induce expression of genes involved in extracellular matrix remodeling such as (*FN1, PLAU1,* and *MMP9*). Moreover, the upregulated expression of immunosuppressive factors such as *IDO1* indicates that transformed epithelial cells can protect themselves from immunosurveillance giving rise to a tumor microenvironment. Even though inflammatory stimuli are predicted propagate the *C. parvum* induced carcinogenesis, PI3K/AKT signaling pathway has been proposed to initiate cancer. Image created using biorender.com.

- Persistent *C. parvum* infection gives rise to an immunosuppressive tumor microenvironment.

Conclusions and Perspectives

In perspective, this body of work opens new avenues to further strengthen the research on *C. parvum* and host interactions to tackle the prevalence and impact of this parasite on human and animal health and more significantly on cancer development.

Future perspectives of these work are as follows:

- One of the main objectives of epigenetic mechanisms is regulations of gene expression. Apart from the regulation of genes necessary for cellular differentiation, epigenetic mechanisms have also been observed to control virulence factors (Hakimi MA and Dietsch KW 2007). The use techniques such as chromatin-immunoprecipitation sequencing (ChIP-seq) can be employed to further functionally analyze histone methylation marks such as H3K4, H3K36, and H4K20 in *C. parvum*. It would be of interest to investigate whether secreted proteins such as Rhoptry protein kinase orthologs of *C. parvum* are regulated by these mechanisms. ROP proteins have been secreted by *T. gondii* to evade the IFNγ-stimulated host immune response (Zhu W et al. 2019) (Figure 10).

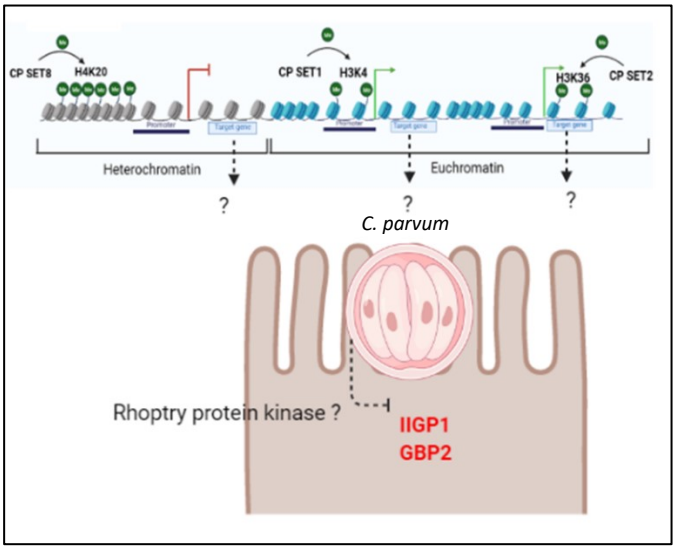


Figure 10. Diagram of potential perspective highlighting epigenetic modulations in the parasite involved in evading host innate immune response. ChIP-seq technique has been proposed to identify the genes of interest which might be regulated by H3K4, H3K36, and H4K20 methylations. And further,r identify whether any of these molecules would be regarded as virulence factors such as rhoptry protein kinases secreted by the parasite to evade the innate immune response by inhibiting the function of IFNγ-stimulated molecules such as IIGP1 and GBP2. Chip-seq, chromatin-immunoprecipitation sequencing. Image created using biorender.com.

Conclusions and Perspectives

- Since we identified the participation of histone modifications during the life cycle of the parasite, pharmacological inhibition of these modifications could be tested to study the consequence in the life cycle of the parasite.

- Despite its impact on public health, no vaccine or chemoprophylactic drugs to prevent *Cryptosporidium* infection and very few chemotherapeutic options for its treatment are currently available (Checkley W et al. 2015). In this respect, commercially available inhibitors of epigenetic regulators such as HDACs have previously been shown to be effective against *C. parvum* (Guo F et al. 2018b). HKMT enzymes regulating methylation marks are responsible for a pan of genes involved in the development of the parasite. Thus, they may be considered as potential targets to curb the infection opening new avenues for anti-parasite drug discovery.

- The role of EMT inducing signaling pathways such as PI3K, MAPK, and NFκβ needs to be further investigated. Moreover, PI3K/AKT signaling pathways have been proposed to epigenetically regulate EMT associated genes in gastric cancer (Liu X et al. 2016). Thus, it would be of interest to determine if there is any potential link between the PI3K/AKT signaling pathway activated as a result of *C. parvum* infection and the dysregulation of epigenetic markers such as H3K27 and H3K36 in the induction of EMT. To validate the preliminary results and this proposed mechanism to be involved in *C. parvum* induced carcinogenesis, inhibitors of key molecules of the PI3K/AKT pathway such as p85α and AKT can be used in *in vitro* and *in vivo* models to analyze the effects on infection and cancer onset (Figure 11).

Conclusions and Perspectives

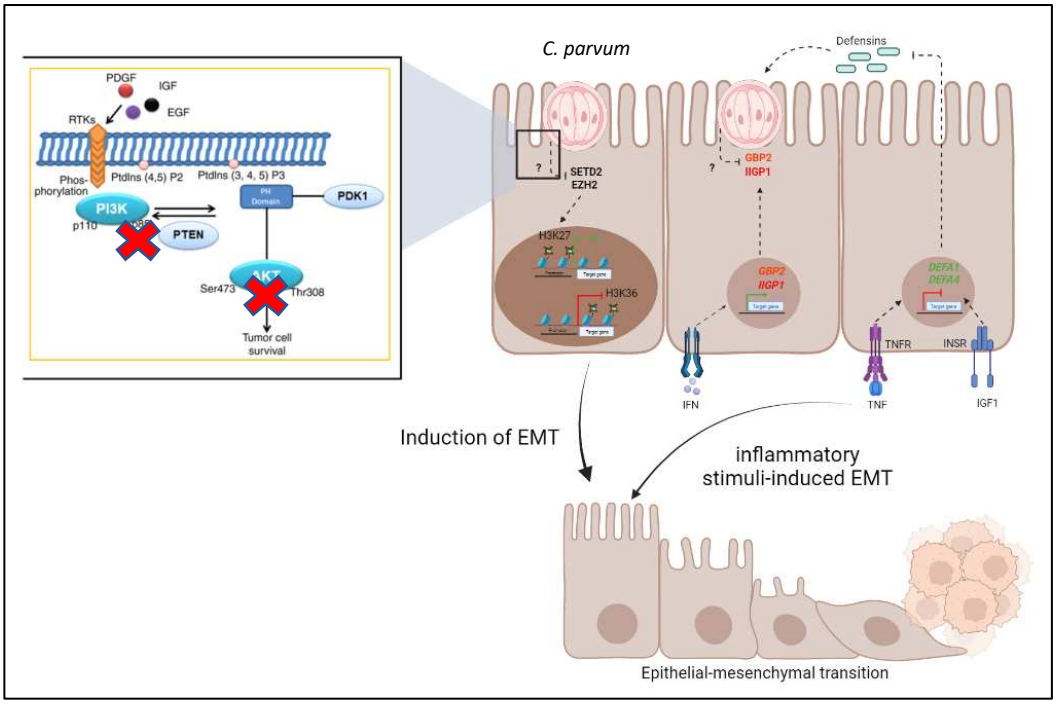


Figure 11. Diagram representing potential crosstalk between PI3K/AKT pathway and epigenetic modifications of the host which lead to *C. parvum* induced EMT. The use of inhibitors of p85α, a regulatory subunit of PI3K complex, and AKT molecules (red cross) has been proposed to validate the role of this pathway at the intersection of *C. parvum* infection and cancer onset. Moreover, investigate whether PI3K/AKT pathway regulates genes involved in EMT induction through epigenetic modifications such as H3K27 or H3K36 methylations by performing Chip-seq analysis in parallel to inflammatory stimuli-induced EMT in our experimental mouse model. Chip-seq, Chromatin immunoprecipitation sequencing; EMT, Epithelial to mesenchymal transition. Image created using biorender.com.

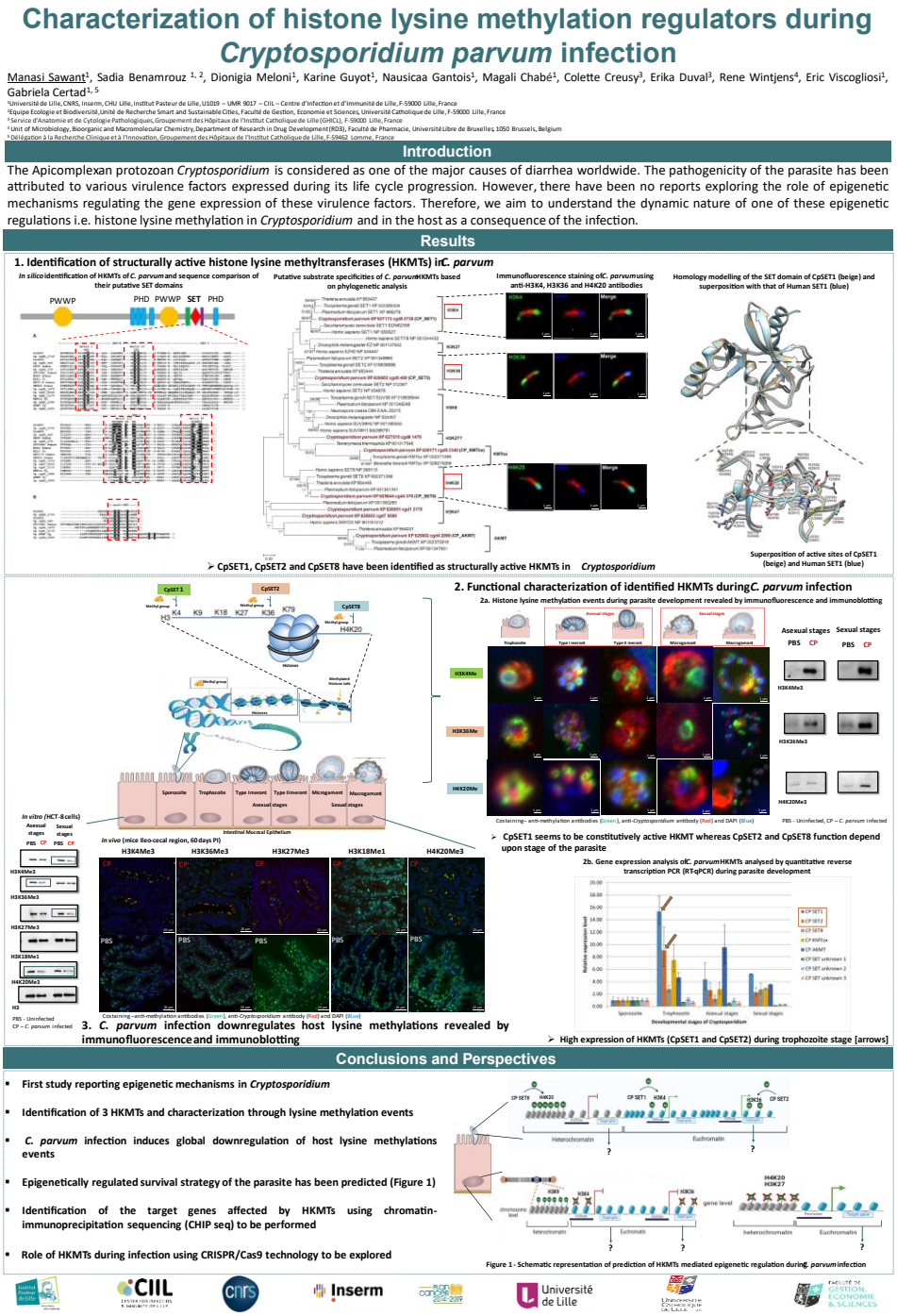
- Single-cell genomics approach can be one of the future perspectives to investigate *C. parvum* induced cellular transformation of intestinal epithelial cells.

6. Appendix

6.1. Work presented at scientific conferences

Symposium day, Centre of Infection and Immunity of Lille (CIIL), Institute Pasteur de Lille, Lille, France, (28th September 2021).

- **Prize: Won 3rd place in poster presentation competition**



Appendix

13th European Multicolloquium of Parasitology, Belgrade, Serbia. (12th-16th October 2021). (Virtual conference)

"First characterization of the role of epigenetics in the dynamics of *Cryptosporidium parvum* infection".

M. SAWANT[1], S. BENAMROUZ [1, 2], D. MELONI[1], K. GUYOT[1], N. GANTOIS[1], M. CHABE[1], C. CREUZY[3], E. DUVAL[3], R. WINTJENS[4], E. VISCOGLIOSI[1], G. CERTAD[1, 5]

[1] manasi.sawant@pasteur-lille.fr, Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France
[2]Equipe Ecologie et Biodiversité, Unité de Recherche Smart and Sustainable Cities, Faculté de Gestion, Economie et Sciences, Institut Catholique de Lille, France
[3] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France
[4] Unit of Microbiology, Bioorganic and Macromolecular Chemistry, Department of Research in Drug Development (RD3), Faculté de Pharmacie, Université Libre de Bruxelles, 1050 Brussels, Belgium
[5] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France

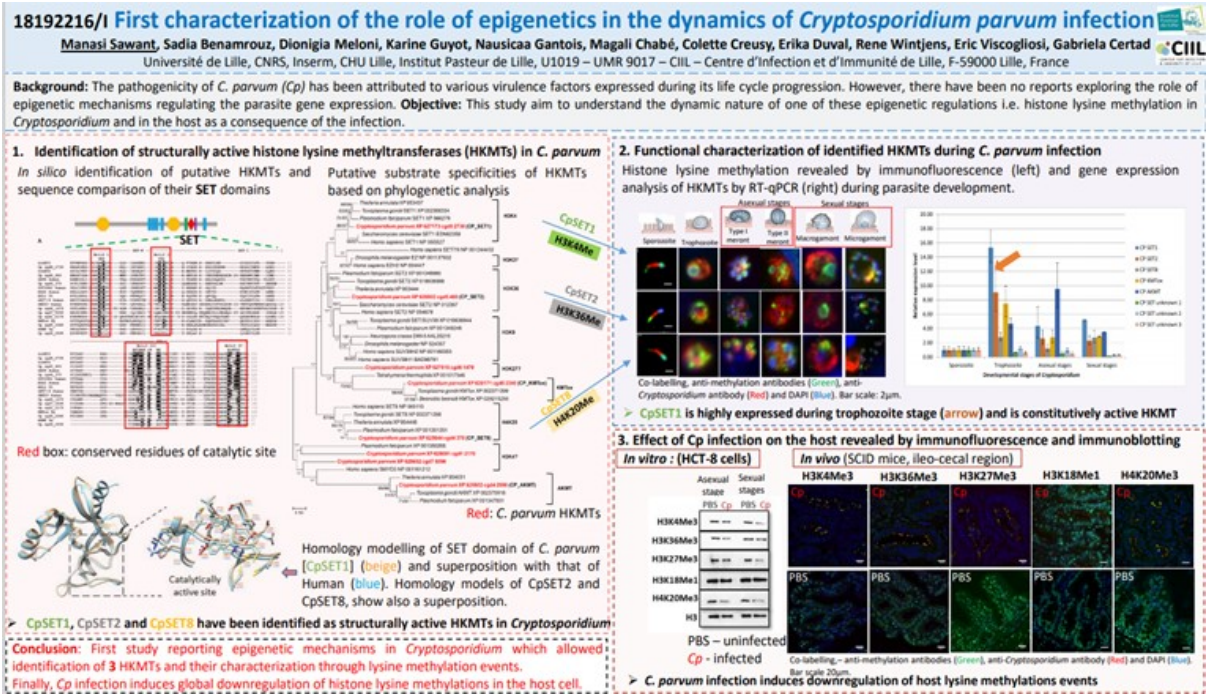
Background. Epigenetic mechanisms are known to be targeted by pathogens to hijack cellular host functions during infection

Objectives. Our aim was thus to explore the role of epigenetics and in particular of histone lysine methylation in *Cryptosporidium parvum* and in the host.

Material and Methods. *In silico* analysis was performed to identify epigenetic regulators. Phylogenetic analysis allowed prediction of substrate specificities of identified lysine methyltransferases (KMTs). Gene expression profile of the identified KMTs was performed by RT-qPCR. Immunofluorescence analysis using antibodies recognizing specific methylated-lysine modifications in the parasite as well as in the host was performed.
Results. 11 putative KMTs were identified. Subsequent alignment of the SET-domain sequences of these KMTs classified the predicted *C. parvum* KMTs into 5 subfamilies: CP SET1, CP_SET2, CP_SET8, CP_KMTox and CP_AKMT. CP_SET1, CP_SET2 and CP_SET8 are predicted as histone lysine methyltransferases (HKMTs) while CP_KMTox and CP_AKMT have been identified as KMTs and exclusively found in Apicomplexa. No evidence of histones lysine demethylases was observed. Phylogenetic analysis confirmed the classification of the 5 subfamilies of *C. parvum* KMTs and their associated putative substrate specificities. Site specific methylation at lysine 4 (K4) and K36 of histone H3 and K20 of histone H4 in sporozoite stage of *C. parvum* confirmed substrate specificities of the HKMTs. Gene expression profile of these putative KMTs during different stages of the parasite development was compared. HKMTs (CP_SET1, CP_SET2) were shown to be highly expressed during the trophozoite stage. Consistently, the specific histone lysine marks displayed dynamic changes during the parasite development. Furthermore, we showed that the infection induces global downregulation of the histone lysine methyl marks in the host cell.
Conclusion. This study highlights the importance of epigenetic mechanisms in gene regulation of virulence factors of *Cryptosporidium* and the potential of this parasite to exploit host epigenetic regulation to its advantage.

Keywords: *Cryptosporidium*; Infection; Epigenetics.

Appendix


18192216/I First characterization of the role of epigenetics in the dynamics of Cryptosporidium parvum infection
Manasi Sawant, Sadia Benamrouz, Dionigia Meloni, Karine Guyot, Nausicaa Gantois, Magali Chabé, Colette Creusy, Erika Duval, Rene Wintjens, Eric Viscogliosi, Gabriela Certad
Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France
CIIL
Background: The pathogenicity of C. parvum (Cp) has been attributed to various virulence factors expressed during its life cycle progression. However, there have been no reports exploring the role of epigenetic mechanisms regulating the parasite gene expression. Objective: This study aim to understand the dynamic nature of one of these epigenetic regulations i.e. histone lysine methylation in Cryptosporidium and in the host as a consequence of the infection.
1. Identification of structurally active histone lysine methyltransferases (HKMTs) in C. parvum
In silico identification of putative HKMTs and sequence comparison of their SET domains
SET
Red box: conserved residues of catalytic site
Putative substrate specificities of HKMTs based on phylogenetic analysis
CpSET1
H3K4Me
CpSET2
H3K36Me
CpSET8
H4K20Me
Red: C. parvum HKMTs
Catalytically active site
Homology modelling of SET domain of C. parvum [CpSET1] (beige) and superposition with that of Human (blue). Homology models of CpSET2 and CpSET8, show also a superposition.
CpSET1, CpSET2 and CpSET8 have been identified as structurally active HKMTs in Cryptosporidium
Conclusion: First study reporting epigenetic mechanisms in Cryptosporidium which allowed identification of 3 HKMTs and their characterization through lysine methylation events.
Finally, Cp infection induces global downregulation of histone lysine methylations in the host cell.
2. Functional characterization of identified HKMTs during C. parvum infection
Histone lysine methylation revealed by immunofluorescence (left) and gene expression analysis of HKMTs by RT-qPCR (right) during parasite development.
Asexual stages
Sexual stages
Sporozoite
Trophozoite
Type I meront
Type II meront
Microgamont
Microgamont
Co-labelling, anti-methylation antibodies (Green), anti-Cryptosporidium antibody (Red) and DAPI (Blue). Bar scale: 2µm.
CpSET1 is highly expressed during trophozoite stage (arrow) and is constitutively active HKMT
3. Effect of Cp infection on the host revealed by immunofluorescence and immunoblotting
In vitro : (HCT-8 cells)
In vivo (SCID mice, ileo-cecal region)
Asexual stage
Sexual stages
PBS Cp PBS Cp
H3K4Me3
H3K36Me3
H3K27Me3
H3K18Me1
H4K20Me3
H3
H3K4Me3
H3K36Me3
H3K27Me3
H3K18Me1
H4K20Me3
PBS – uninfected
Cp - infected
Co-labelling,– anti-methylation antibodies (Green), anti-Cryptosporidium antibody (Red) and DAPI (Blue).
Bar scale 20µm.
C. parvum infection induces downregulation of host lysine methylations events

Appendix

13th European Multicolloquium of Parasitology, Belgrade, Serbia. (12th-16th October 2021). (Virtual conference)

***Cryptosporidium parvum* infection and colon cancer. Results of a microarray approach.**

M. Sawant[1], Sadia Benamrouz[1,2], Anthony Mouray[3], Nausicaa Gantois[1], Colette Creusy[4], Erika Duval[4], Adriana Mihalache[4], Pierre Gosset [4], David Hot[1], Eric Viscogliosi[1], Gabriela Certad[1, 5]

[1] manasi.sawant@pasteur-lille.fr Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France
[2] Laboratoire Ecologie et Biodiversité, Faculté de Gestion Economie et Sciences, Institut Catholique de Lille, F-59000 Lille, France.
[3] Plateforme d'Expérimentations et de Hautes Technologies Animales, Institut Pasteur de Lille, Lille, France.
[4] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France.
[5] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France.

Background. Accumulative experimental and clinical evidences link *Cryptosporidium parvum* infection and digestive adenocarcinoma.

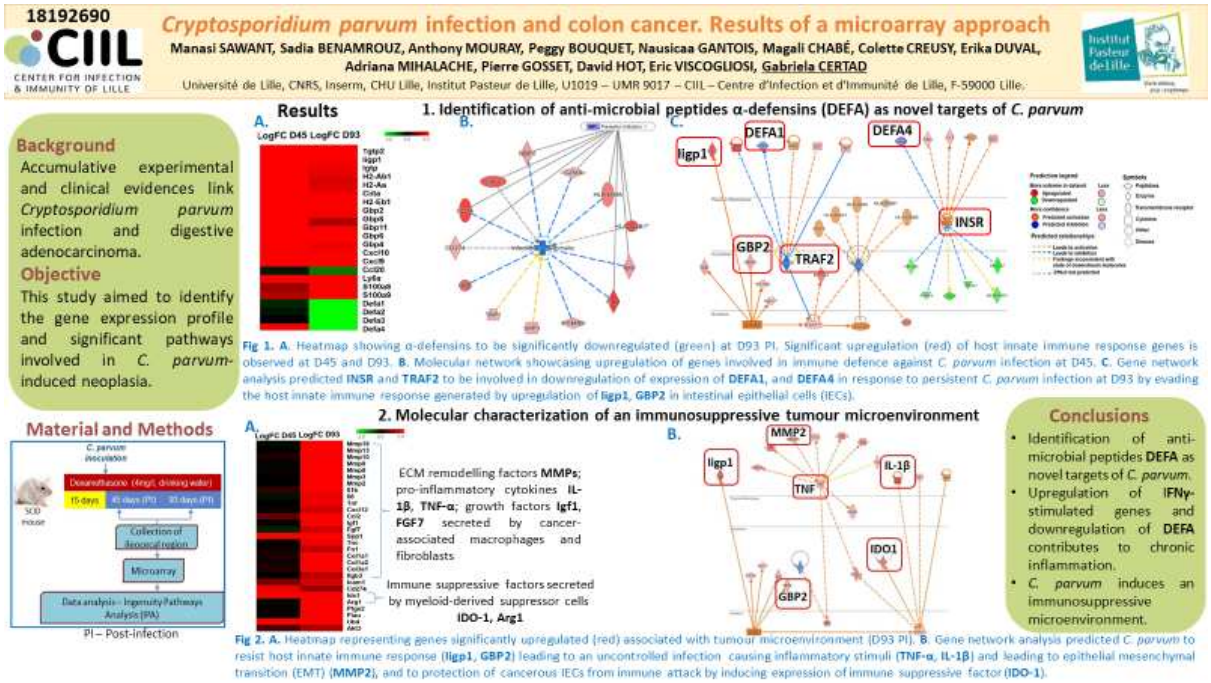
Objective. This study aimed to identify the gene expression profile and significant pathways involved in *C. parvum*-induced neoplasia.

Material and Methods. 24 SCID mice were divided into 4 groups: 1) uninfected and 2) infected at 45 days post-infection (PI), 3) uninfected and 4) infected at 93 days PI . Histopathology and Agilent SurePrint G3 Mouse microarray analysis were performed. Ingenuity Pathway Analysis (IPA) and gProfiler allowed pathway analyzes. Microarray data was validated by RT-qPCR.
Results. The gene expression profile was significantly altered with 92 and 755 genes upregulated at 45 and 93 days PI, respectively, and 39 and 303 downregulated at 45 and 93 days PI, respectively (logFC 2.0). IPA analysis for Group 4 identified 27/173 genes of the tumor microenvironment pathway (z score >2). The software also annotated 166 genes from the dataset corresponding to 14 different functions associated with cancer (z score >2). Molecular gene network built using highly regulated genes such as Defa1 and IDO-1 predicted the role of Paneth cells, which are specialized intestinal epithelial cells present in the crypts of small intestine to be involved in parasite infection propagation and tumor progression. Either at 45 or 93 days PI, genes were significantly enriched in the biological process of the immunological response, and the cellular component indicated that these genes were predominantly located in the extracellular region, membrane and cell surface. As for molecular function, these genes were enriched in GTP binding at 45 days PI, and signaling receptor and integrin binding and 93 days PI. The most significant KEGG pathways were associated with cytokine-cytokine and ECM receptors interaction.

Conclusion. In the current study, we identified for the first time the alteration in the gene expression profile of the *C. parvum*-induced neoplasia.

Keywords: *Cryptosporidium*; Transcriptomics; Cancer

Appendix


18192690
CIIL
CENTER FOR INFECTION & IMMUNITY OF LILLE
Cryptosporidium parvum infection and colon cancer. Results of a microarray approach
Manasi SAWANT, Sadia BENAMROUZ, Anthony MOURAY, Peggy BOUQUET, Nausicaa GANTOIS, Magali CHABÉ, Colette CREUSY, Erika DUVAL, Adriana MIHALACHE, Pierre GOSSET, David HOT, Eric VISCOGLIOSI, Gabriela CERTAD
Université de Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille.
Institut Pasteur de Lille
Background
Accumulative experimental and clinical evidences link Cryptosporidium parvum infection and digestive adenocarcinoma.
Objective
This study aimed to identify the gene expression profile and significant pathways involved in C. parvum-induced neoplasia.
Material and Methods
C. parvum inoculation
Dexamethasone (4mg/L drinking water)
SCID mouse
15 days
45 days (PI)
90 days (PI)
Collection of ileocecal region
Microarray
Data analysis – Ingenuity Pathways Analysis (IPA)
PI – Post-Infection
Results
1. Identification of anti-microbial peptides α-defensins (DEFA) as novel targets of C. parvum
A.
LogFC D45 LogFC D93
B.
C.
DEFA1
DEFA4
Iigp1
GBP2
TRAF2
INSR
Fig 1. A. Heatmap showing α-defensins to be significantly downregulated (green) at D93 PI. Significant upregulation (red) of host innate immune response genes is observed at D45 and D93. B. Molecular network showcasing upregulation of genes involved in immune defence against C. parvum infection at D45. C. Gene network analysis predicted INSR and TRAF2 to be involved in downregulation of expression of DEFA1, and DEFA4 in response to persistent C. parvum infection at D93 by evading the host innate immune response generated by upregulation of Iigp1, GBP2 in intestinal epithelial cells (IECs).
2. Molecular characterization of an immunosuppressive tumour microenvironment
A.
LogFC D45 LogFC D93
ECM remodelling factors MMPs; pro-inflammatory cytokines IL-1β, TNF-α; growth factors Igf1, FGF7 secreted by cancer-associated macrophages and fibroblasts
Immune suppressive factors secreted by myeloid-derived suppressor cells IDO-1, Arg1
B.
MMP2
Iigp1
IL-1β
TNF
IDO1
GBP2
Conclusions
• Identification of anti-microbial peptides DEFA as novel targets of C. parvum.
• Upregulation of IFNγ-stimulated genes and downregulation of DEFA contributes to chronic inflammation.
• C. parvum induces an immunosuppressive microenvironment.
Fig 2. A. Heatmap representing genes significantly upregulated (red) associated with tumour microenvironment (D93 PI). B. Gene network analysis predicted C. parvum to resist host innate immune response (Iigp1, GBP2) leading to an uncontrolled infection causing inflammatory stimuli (TNF-α, IL-1β) and leading to epithelial mesenchymal transition (EMT) (MMP2), and to protection of cancerous IECs from immune attack by inducing expression of immune suppressive factor (IDO-1).

Appendix

13th European Multicolloquium of Parasitology, Belgrade, Serbia. (12th-16th October 2021). (Virtual conference)

Molecular epidemiology of *Blastocystis* amongst Syrian refugee communities living in North Lebanon

S KHALED[1,2], M. SAWANT[1], N. GANTOIS[1], A. AYOUBI[2], G EVEN[3], J EL HOUMAYRAA[4], M NABOT[5], S BENAMROUZ[1], M CHABE[1], G CERTAD[1], D EL SAFADI[2], F DABBOUSSI[2], M HAMZE[2], E. VISCOGLIOSI[1]

[1] manasi.sawant@pasteur-lille.fr, Institut Pasteur of Lille, Center for Infection and Immunity, Inserm U1019, UMR CNRS 9017, University of Lille, CHU of Lille, Lille, France
[2] Université Libanaise, Tripoli, Lebanon
[3] Gènes Diffusion, Douai, France
[4] Concern Worlwide, Akkar, Lebanon
[5] Solidarités International, Beirut, Lebanon

Background. Several geographical regions around the world such as the Middle East remain yet poorly explored regarding the molecular epidemiology of *Blastocystis*.
Objectives. We performed the first epidemiological survey ever conducted in the Syrian refugee population living in North Lebanon. Since molecular data regarding *Blastocystis* epidemiology were already available from the local Lebanese population, our second aim was to compare the subtypes (STs) and genotypes identified in the Syrian and local populations to investigate the circulation of the parasite between both communities.

Material and Methods. A total of 306 stool samples were collected from Syrian refugees living in 26 Informal Tented Settlements (ITS) subjected or not to Water, Sanitation and Hygiene (WASH) interventions in North Lebanon, then screened for the presence of *Blastocystis* by real-time polymerase chain reaction targeting the small subunit RNA gene followed by subtyping.

Results. The overall prevalence of the parasite reached 63.7% and *Blastocystis* colonization was not significantly associated with gender, age, symptomatic status, abdominal pain or diarrhea. In contrast, WASH intervention status of ITS was identified as a risk factor for infection. Among a total of 164 subtyped isolates, ST3 was predominant, followed by ST1, ST2 and ST10. No particular ST was reported to be associated with age, gender, symptomatic status or WASH intervention status of ITS. Intra-ST diversity of ST1 to ST3 was low suggesting large-scale anthroponotic transmission. Moreover, few ST1 to ST3 genotypes were common to the Syrian refugees and host populations.

Conclusion. The high prevalence observed in the Syrian cohort highlights the active circulation of the parasite in this population in link with poor sanitation conditions. *Blastocystis* is mainly transmitted through the inter-human route in the Syrian cohort and the circulation of the parasite between the refugee and host communities remains limited because of reduced contact between these two populations.

Keywords: *Blastocystis*; Molecular epidemiology; Transmission.

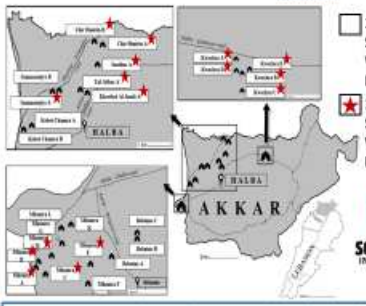
Appendix

18192216/II Prevalence and subtype distribution of *Blastocystis* amongst Syrian refugee communities living in North Lebanon

Salma Khaled, Manasi Sawant, Nausicaa Gantois, Aisha Ayoubi, Gaël Even, Jinane El Houmayraa, Mathieu Nabot, Sadia Benamrouz-Vanneste, Magali Chabé, Gabriela Certad, Dima El Safadi, Fouad Dabboussi, Monzer Hamze, Eric Viscogliosi
Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France

Molecular data concerning the prevalence and subtype (ST) distribution of the intestinal parasite *Blastocystis* remain scarce in the Middle East. Therefore, our first aim was to fill a geographical gap in the knowledge of *Blastocystis* prevalence and diversity by conducting a large-scale epidemiological survey in Syrian refugees in North Lebanon and to assess the burden of the parasite in this at-risk population. In addition, since molecular data regarding *Blastocystis* epidemiology were already available in the North Lebanese population, our second aim was thus to compare the STs and genotypes identified in the Syrian and local populations to investigate the circulation/transmission of the parasite between both communities.

1) Assessing the burden of *Blastocystis* in the at-risk population of Syrian refugees in North Lebanon



☐ 16 Informal Tented Settlements (ITS) With WASH intervention (WI); N = 184 stool samples

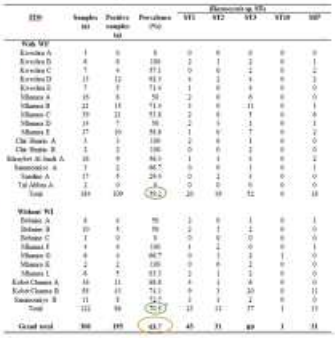
☆ 10 Informal Tented Settlements (ITS) Without WASH intervention (WI); N = 122 stool samples

SOLIDARITÉS INTERNATIONAL — CONCERN worldwide

Screening by real-time PCR followed by sequencing of the positive PCR samples

- ✓ Overall prevalence of 63.7%
- ✓ Prevalence significantly higher in ITS without WI
- ✓ Prevalence not significantly higher in symptomatic refugees and not associated with gender or age

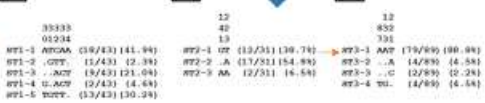
2) Comparing STs and genotypes identified in the Syrian and local populations



Distribution of STs among the Syrian population

- ✓ ST3 > ST1 > ST2 > ST10 among single infections
- ✓ ST distribution not associated with gender, age, symptomatic status of refugees or status of ITS

A → B → C



Intra-ST diversity and identification of Syrian genotypes

- ✓ Low number of genotypes
- ✓ Low intra-ST diversity of ST1 to ST3
- → Large-scale anthroponotic transmission among refugees within ITS

Comparative analysis of genotypes identified in the Syrian and North Lebanese populations

- ✓ Only 16.6% of the 277 Lebanese ST1 to ST3 isolates classified among the 12 Syrian genotypes
- ✓ Predominant Syrian genotype ST3-1 not identified in the Lebanese population
- ✓ Restricted circulation of the parasite between local and refugee populations because of limited contact

Appendix

13th European Multicolloquium of Parasitology, Belgrade, Serbia. (12th-16th October 2021). (Virtual conference)

Molecular epidemiology of trichomonads in wild wetland birds in the Netherlands

W.J.M. LANDMAN[1], M. SAWANT[2], N. GANTOIS[2], F.A. MAJOOR[3], J.H.H. VAN ECK[4], E. VISCOGLIOSI[2]

[1] Royal GD, Deventer, Netherlands
[2] manasi.sawant@pasteur-lille.fr, Institut Pasteur of Lille, Center for Infection and Immunity, Inserm U1019, UMR CNRS 9017, University of Lille, CHU of Lille, Lille, France
[3] SOVON Vogelonderzoek Nederland, Nijmegen, Netherlands
[4] Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands

Background. Severe granulomatosis in productive layers due to *Tetratrichomonas gallinarum* strain 13/16632 infection occurred in 2013 and 2017 on farms situated in a wetland area in the Netherlands. These outbreaks were mainly characterized by persistent increased mortality of hens and by a high within flock incidence of granulomas. **Objectives.** Our aim was to evaluate the potential of wild wetland birds to act as a reservoir of virulent trichomonads such as *T. gallinarum* especially for chicken rearing farms.

Material and Methods. A prevalence survey on trichomonads was performed by analysing cloaca swabs of 526 birds belonging to 13 species of wetland birds. The number of birds sampled ranged from 1 to 275 per species. Birds were sampled at 15 locations, distributed over the Netherlands. DNA extracted from the cloaca swabs was subjected to a nested PCR assay using trichomonad specific primers targeting the ITS1 – 5.8S rRNA – ITS2 region. Positive nested PCR products were either cloned before sequencing or directly sequenced.

Results. Trichomonads were detected in nine bird species. The overall prevalence was 9% (47/526), while the prevalence in the five species of which a substantial number of birds were examined (at least 39 per species) ranged from 4 to 24%. Three trichomonad species were found: *T. gallinarum*, *Trichomonas tenax* and *Simplicimonas* sp. of which *T. gallinarum* dominated. The virulent *T. gallinarum* strain 13/16632 was not detected, but closely related strains were identified. Phylogenetic analysis revealed that all *T. gallinarum* isolates belonged to two clusters within lineage 15 of *Tetratrichomonas* lineages. All *T. tenax* isolates were identical and clustered with reference strains, while *Simplicimonas* sp. isolates showed large genetic diversity. Some isolates may represent a new species of the genus *Simplicimonas*.

Conclusion. We highlight that trichomonads are widespread and circulate abundantly amongst wetland birds, questioning, amongst others, its relevance for commercial poultry.

Keywords: Molecular epidemiology; Trichomonads; Wild wetland birds

Appendix

18192216/III Molecular epidemiology of trichomonads in wild wetland birds in the Netherlands

Landman WJM[1], Sawant M[2], Gantois N[2], Majoor FA[1], van Eck JHH[1], Viscogliosi E[2]
[1] Royal GD, Deventer, Netherlands
[2] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille , Lille, France

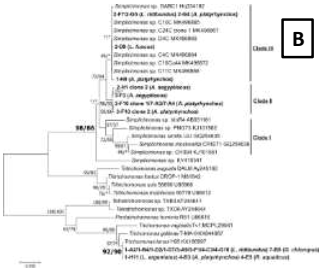
Severe granulomatosis in productive chicken layers due to *Tetratrichomonas gallinarum* strain 13/16632 infection occurred in 2013 (Landman et al. 2016 Avian Pathol) and 2017 (Landman et al. 2019 Vet Quart) on farms situated in a wetland area in the Netherlands. We hypothesized that wetland birds could be the source of the infection. Therefore, a large-scale prevalence study on trichomonads was performed by analysing cloaca swabs of wetland birds in the Netherlands using molecular methods.

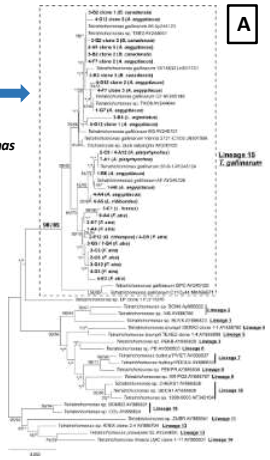
Map showing the geographic locations of the sampling. The size of circles is proportional to the number of birds caught (green area, number of trichomonad negative birds; red area, number of birds positive for trichomonads)

- ✓ Study conducted between August 2019 and March 2020 at 15 locations distributed over the Netherlands
- ✓ Sampling of 526 cloaca swabs from 13 different wetland bird species predominantly captured in city parks and river quays

DNA extraction from cloaca swabs
Screening by nested PCR targeting the ITS1 -5.8S-ITS2 gene
Direct sequencing or cloning of the positive PCR samples
Phylogenetic analyses

- ✓ Overall prevalence of trichomonads of 9% (47/526)
- ✓ Molecular characterization of 55 trichomonad isolates through phylogenetic analyses identified as *Tetratrichomonas gallinarum* , *Trichomonas tenax* and *Simplicimonas*





Unrooted Neighbor-joining trees based on the ITS1-5.8S-ITS2 sequences. A. All tetratrichomonad sequences obtained in this study (in bold) belong to the Tetratrichomonas *lineage 15 corresponding to* T. gallinarum. *B. All other new trichomonad sequences (in bold) group with* Simplicimonas *and* T. tenax

- ❑ Trichomonads frequently occur among wild wetland birds in the Netherlands
- ❑ Identification of *T. gallinarum* , *T. tenax* and *Simplicimonas* sp., with a large predominance of *T. gallinarum*
- ❑ Some *T. gallinarum* isolates are closely related to a strain causing granulomas in chicken layers
- ❑ Some isolates may represent a new species of the genus *Simplicimonas*

Appendix

15 International Workshop on Opportunistic Protists, České Budějovice, Czech Republic. (15th-17th June 2021).

Virtual conference

Oral presentation

First study to characterize the role of epigenetics in the biology of the Apicomplexan parasite *Cryptosporidium parvum*

Manasi Sawant[1], Sadia Benamrouz [1, 2], Dionigia Meloni[1], Karine Guyot[1], Nausicaa Gantois[1], Magali Chabé[1], Colette Creusy[3], Erika Duval[3], Jonathan Weitzman[4], Eric Viscogliosi[1], Gabriela Certad [1, 5]

[1] Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France
[2] Laboratoire Ecologie et Biodiversité, Faculté de Gestion Economie et Sciences, Institut Catholique de Lille, F-59000 Lille, France.
[3] Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), F-59000 Lille, France.
[4] Université de Paris, UMR 7216 Epigenetics and Cell Fate, CNRS, Paris, France
[5] Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France.

INTRODUCTION: The role of epigenetic mechanisms known to be targeted by other pathogens in order to hijack cellular host functions, are unexplored concerning *Cryptosporidium* infection. OBJECTIVE: To explore the role of epigenetics and in particular of histone lysine methylation in *Cryptosporidium* and in the host. MATERIAL AND METHODS: *In silico* analysis for the identification of epigenetic players was performed. Phylogenetic analysis allowed the identification of putative substrate specificities of the lysine methylation regulators. Analysis of the gene expression of these regulators was performed by RT-PCR during *Cryptosporidium* infection *in vitro*. Immunofluorescence analysis of *Cryptosporidium*-infected cells with antibodies recognizing specific methylated-Lysine modifications in the parasite as well as in the host was performed. RESULTS: *In silico*, 11 putative lysine methyltransferases (KMTs) were identified. Further, alignment of the SET-domain sequences of the putative KMTs with the representatives of the SET domain subfamilies classified the predicted *C. parvum* KMTs into 5 subfamilies: CP SET1, CP_SET2, CP_SET8, CP_KMTox and CP_AKMT. CP_SET1, CP_SET2 and CP_SET8 are predicted as histone lysine methyltransferases (HKMTs) while CP_KMTox and CP_AKMT have been identified as KMTs, found exclusively in the phylum Apicomplexa. We found no evidence of histones lysine demethylases. Phylogenetic analysis confirmed the classification of the 5 subfamilies of *C. parvum* KMTs and their associated putative substrate specificities. Site specific methylation at lysine 4 (K4) and K36 of histone H3 and K20 of histone H4 in sporozoite stage of *C. parvum* confirmed substrate specificities of the identified HKMTs. We compared the gene expression profile of these putative KMTs during different stages of the parasite development, to observe HKMTs (CP_SET1, CP_SET2) to be highly expressed during the trophozoite stage of the parasite. Consistently, the specific histone lysine marks also displayed dynamic changes during the parasite development. Furthermore, we showed that the infection induces global downregulation of the histone lysine methyl marks in the host cell. CONCLUSIONS: This study indicates the importance of epigenetic mechanisms in gene regulation of virulence factors of the enteric parasite *Cryptosporidium* and the potential to exploit host epigenetic regulation to its advantage.

Appendix

3rd International Blastocystis conference. Virtual Edition (2nd – 4th June 2021).

Prevalence and subtype distribution of *Blastocystis* sp. amongst Syrian refugee communities living in North Lebanon

Salma Khaled[1,2], Nausicaa Gantois[1], Aisha Ayoubi[2], Gaël Even[3,4], Manasi Sawant[1], Jinane El Houmayraa[5], Mathieu Nabot[6], Sadia Benamrouz-Vanneste[1,7], Magali Chabé[1], Gabriela Certad[1,8] , Dima El Safadi[2], Fouad Dabboussi[2] , Monzer Hamze[2] and Eric Viscogliosi[1]

[1]Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 9017 – CIIL – Centre d'Infection et d'Immunité de Lille, F-59000 Lille, France
[2]Laboratoire Microbiologie Santé Environnement (LMSE), Ecole Doctorale des Sciences et Technologie, Faculté de Santé Publique, Université Libanaise, Tripoli, Lebanon
[3]Gènes Diffusion, F-59501 Douai, France
[4]PEGASE-Biosciences (Plateforme d'Expertises Génomiques Appliquées aux Sciences Expérimentales), Institut Pasteur de Lille, F-59000 Lille, France
[5]Concern Worldwide, HDYS Building (Opposite Abdel Karim Rifai Petrol Station), Halba, Akkar, Lebanon
[6]Solidarités International, Ras Al Naba'a, Chaar street, Chaar building, Beirut, Lebanon
[7]Laboratoire Ecologie et Biodiversité, Faculté de Gestion Economie et Sciences, Institut Catholique de Lille, F-59000 Lille, France
[8]Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, F-59462 Lomme, France

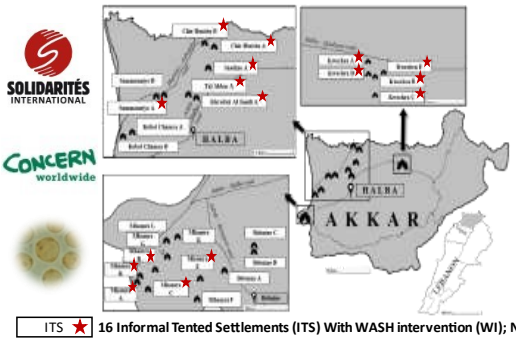
Data concerning the prevalence and subtype (ST) distribution of *Blastocystis* remain scarce in the Middle East. No epidemiological study has ever been performed in the Syrian population, in part because of the humanitarian crisis heavily impacting this country. This crisis has also displaced a huge number of refugees into Lebanon. Most Syrian refugees are at risks for contracting parasitic infections as they face poverty without proper access to infrastructure services and adequate sewage systems. This may obviously create the perfect environment for wide dissemination and outbreaks of parasitic infections among Syrians and Lebanese population as well. A total of 306 stool samples were collected from Syrian refugees living in 26 Informal Tented Settlements (ITS) subjected or not to Water, Sanitation and Hygiene (WASH) interventions in North Lebanon, then screened for the presence of *Blastocystis* by real-time PCR followed by subtyping. The overall prevalence of the parasite reached 63.7%. *Blastocystis* colonization was not significantly associated with gender, age, symptomatic status, abdominal pain or diarrhea. In contrast, WASH intervention status of ITS was identified as a risk factor for infection. Among a total of 164 subtyped isolates, ST3 was predominant, followed by ST1, ST2 and ST10. No particular ST was reported to be associated with age, gender, symptomatic status, digestive disorders or WASH intervention status of ITS. Intra-ST diversity of ST1 to ST3 was low suggesting large-scale anthroponotic transmission. In parallel, STs and genotypes identified in the Syrian cohort and previously in the local population were compared to investigate the circulation of the parasite between both communities. The comparative analysis of ST1 to ST3 genotypes strongly suggested that *Blastocystis* is mainly transmitted through the inter-human route in the Syrian cohort and that the circulation of the parasite between the refugee and host communities remains limited because of restricted contacts between both populations.

Appendix

Prevalence and subtype distribution of *Blastocystis* sp. amongst Syrian refugee communities living in North Lebanon

Manasi SAWANT, Institut Pasteur of Lille, France

1) Assessing the burden of *Blastocystis* sp. in the at-risk population of Syrian refugees in North Lebanon



ITS ★ 16 Informal Tented Settlements (ITS) With WASH intervention (WI); N = 184 stool samples

ITS 10 Informal Tented Settlements (ITS) Without WASH intervention (WI); N = 122 stool samples

Screening by real-time PCR followed by sequencing of the positive PCR samples

- ✓ Overall prevalence of 63.7%
- ✓ Prevalence significantly higher in ITS without WI
- ✓ Prevalence not significantly higher in symptomatic refugees and not associated with gender or age

2) Comparing STs and genotypes identified in the Syrian and local populations

ITS*	Samples (n)	Positive samples (n)	Prevalence (%)	*Blastocystis* sp. STs				
				ST1	ST2	ST3	ST10	MIP
With WI								
Kweches A	1	0	0	0	0	0	0	0
Kweches B	6	6	100	2	1	2	0	1
Kweches C	7	4	57.1	0	0	2	0	2
Kweches D	13	12	92.3	4	2	4	0	2
Kweches E	7	5	71.4	1	0	4	0	0
Mhanra A	16	8	50	2	0	6	0	0
Mhanra B	21	15	71.4	3	0	11	0	1
Mhanra C	39	21	53.8	2	8	5	0	6
Mhanra D	14	7	50	2	3	1	0	1
Mhanra E	17	10	58.8	1	0	7	0	2
Chir Hnaira A	1	1	100	2	0	1	0	0
Chir Hnaira B	2	2	100	0	0	2	0	0
Khayfet Al Jnaeh A	16	9	56.3	1	3	3	0	2
Sanaouniye A	3	2	66.7	0	0	1	0	1
Saadaa A	17	5	29.4	0	2	3	0	0
Tal Abbas A	2	0	0	0	0	0	0	0
Total	184	109	59.2	20	19	52	0	18
Without WI								
Bebnine A	8	4	50	2	0	1	0	1
Bebnine B	10	5	50	2	1	2	0	0
Bebnine C	1	0	0	0	0	0	0	0
Mhanra F	4	4	100	1	2	0	0	1
Mhanra G	6	4	66.7	0	1	2	1	0
Mhanra K	2	2	100	0	0	2	0	0
Mhanra L	6	5	83.3	2	1	2	0	0
Kobet Chamra A	16	11	68.8	4	1	6	0	0
Kobet Chamra B	58	43	74.1	9	3	20	0	11
Sanaouniye B	11	8	72.7	3	3	2	0	0
Total	122	86	70.5	23	12	37	1	13
Grand total	306	195	63.7	43	31	89	1	31

Distribution of STs among the Syrian population

- ✓ ST3 > ST1 > ST2 > ST10 among single infections
- ✓ ST distribution not associated with gender, age, symptomatic status of refugees or status of ITS

Intra-ST diversity and identification of Syrian genotypes

- ✓ Low number of genotypes
- ✓ Low intra -ST diversity of ST1 to ST3
- → Large-scale anthroponotic transmission among refugees within ITS

A

```
          33333
          01234
ST1-1 ATCAA (18/43) (41.9%)
ST1-2 .GTT. (1/43) (2.3%)
ST1-3 ..ACT (9/43) (21.0%)
ST1-4 G.ACT (2/43) (4.6%)
ST1-5 TGTT. (13/43) (30.2%)
```

B

```
          12
          42
          13
ST2-1 GT (12/31) (38.7%)
ST2-2 .A (17/31) (54.8%)
ST2-3 AA (2/31) (6.5%)
```

C

```
          12
          832
          731
ST3-1 AAT (79/89) (88.8%)
ST3-2 ..A (4/89) (4.5%)
ST3-3 ..C (2/89) (2.2%)
ST3-4 TG. (4/89) (4.5%)
```

Comparative analysis of genotypes identified in the Syrian and North Lebanese populations

- ✓ Only 16.6% of the 277 Lebanese ST1 to ST3 isolates classified among the 12 Syrian genotypes
- ✓ Predominant Syrian genotype ST3 -1 not identified in the Lebanese population
- ✓ Restricted circulation of the parasite between local and refugee populations because of limited contact

Appendix

7th International *Giardia* and *Cryptosporidium* Conference, Rouen, France. (24th – 26th June 2019).

"Is *Cryptosporidium parvum* able to induce intestinal neoplasia in vitro?"

Manasi Sawant[1], Martha Baydoun[1], Colette Creusy[2], Nausicaa Gantois[1], Magali Chabé[1], Anthony Mouray[3], Eric Viscogliosi[1], Gabriela Certad[1,4], and Sadia Benamrouz-Vanneste[1,5].

[1]Univ. Lille, CNRS, Inserm, CHU Lille, Institut Pasteur de Lille, U1019 – UMR 8204 – CIIL – Centre d'Infection et d'Immunité de Lille, Lille, France
[2]Service d'Anatomie et de Cytologie Pathologiques, Groupement des Hôpitaux de l'Institut Catholique de Lille (GHICL), Lille, France
[3]Plateforme d'Expérimentations et de Hautes Technologies Animales, Institut Pasteur de Lille, Lille, France
[4]Délégation à la Recherche Clinique et à l'Innovation, Groupement des Hôpitaux de l'Institut Catholique de Lille, Lille, France
[5]Equipe Ecologie et biodiversité, Unité de Recherche Smart and Sustainable Cities, Faculté de Gestion Economie et Sciences, Institut Catholique de Lille, France.

The apicomplexan parasite, *Cryptosporidium,* is a major public health problem in humans and animals. The Global Burden of Disease 2015 Study outlines that *Cryptosporidium* is the second leading cause of death due to diarrhea in children under 5 years old. Additionally, our team has reported that *Cryptosporidium parvum* can induce the development of invasive gastrointestinal neoplasia in an immunocompromised rodent model. We also found that *Cryptosporidium* spp was detected significantly more often in colonic biopsies of patients with colon adenocarcinoma before any treatment than in the control group. However, the study of the pathophysiology of this parasite, which is of crucial interest for the understanding of its link with carcinogenesis is hampered by the lack of a continuous *in vitro* culture system. Recently, we developed a three-dimensional (3D) culture model from adult SCID mice colon that allowed the completion of the parasite life cycle with production of newly formed oocysts. Then, having this system available, the following two questions were investigated: (i) is *C. parvum* able to induce neoplasia in an *in vitro* system in which there is no influence of the host immune response? (ii) is *C. parvum* able to develop and induce neoplasia in a 3D culture system based on colon explants from immunocompetent mice?. For answer these questions, we performed a 3D culture using colon explants from either adult SCID or wild type C57BL/6JRj mice that were co-cultured with 25 excysted C*. parvum* oocysts. Culture explants were stopped periodically and subsequently analyzed histologically. Strikingly, we noted the development of low-grade intraepithelial neoplasia as soon as 27 days post-infection in both systems conceived with colon explants from either immunosuppressed or immunocompetent mice. These results are very interesting as they provide new evidences of the carcinogenic power of *C. parvum,* confirming observations obtained previously in the animal model and in epidemiological studies. In addition*,* it seems that the host immune response is not involved in the mechanisms of *C. parvum*-induced carcinogenesis. This topic should be further investigated as we know, for now on, that around 20% of human cancers have infectious origin. Consequently, if the causal link between *Cryptosporidium* and cancer is clearly established in human tissues, a significant number of digestive cancers could be prevented.

Appendix

6.2. Other articles published in peer-reviewed international journals

Article 1:

Gabriela Certad, Alireza Zahedi, Nausicaa Gantois, **Manasi Sawant**, Colette Creusy, Erika Duval, Sadia Benamrouz-Vanneste, Una Ryan, Eric Viscogliosi (2020) Molecular Characterization of novel *Cryptosporidium* fish genotypes in edible marine fish. *Microorganisms 8, 2014*

Contribution of the student to the study

- Performing microscopy experiments
- Interpretation of the results

Abstract

Current knowledge of *Cryptosporidium* species/genotypes in marine fish is limited. Following phylogenetic analysis at the 18S rDNA locus, a recent study identified six new genotypes of *Cryptosporidium* colonizing edible fish found in European seas. Of these, five grouped in a clade together (#Cryptofish 1-5) and one grouped separately (#Cryptofish 7). In the present study, after phylogenetic analyzes of #Cryptofish1, #Cryptofish2, #Cryptofish4, #Cryptofish5 and #Cryptofish7 at the actin locus, the presence of two major clades was confirmed. In addition, when possible, longer 18S amplicons were generated. In conclusion, the small genetic distances between these genotypes designated as a novel marine genotype I (#Cryptofish 1-5) suggest that they may be genetic variants of the same species, while the designated novel marine genotype 2 (#Cryptofish 7) is clearly representative of a separate species.

Appendix

Article 2:

Salma Khaled, Nausicaa Gantois, Amadou Tidjani Ly, Simon Senghor, Gaël Even, Ellena Dautel, Romane Dejager, **Manasi Sawant**, Martha Baydoun, Sadia Benamrouz-Vanneste, Magali Chabé, Seynabou Ndiaye, Anne-Marie Schacht, Gabriela Certad, Gilles Riveau, Eric Viscogliosi (2020) Prevalence and subtype distribution of *Blastocystis* sp. in Senegalese school children. *Microorganisms 8, 1408*

Contribution of the student to the study

- Sequence analysis and alignment of the obtained sequences
- Interpretation of the data obtained

Abstract

Blastocystis sp. is an enteric protozoan that frequently colonizes humans and many animals. Despite impacting on human health, data on the prevalence and subtype (ST) distribution of *Blastocystis* sp. remain sparse in Africa. Accordingly, we performed the first multicenter and largest epidemiological survey ever conducted on *Blastocystis* sp. for this continent. A total of 731 stool samples collected from healthy school children living in 10 villages of the northwestern region of Senegal were tested for the presence of *Blastocystis* sp. by real-time polymerase chain reaction followed by subtyping of positive samples. Considerable variation in prevalence between villages (51.7 to 100%) was evident with the overall prevalence being 80.4%. Mixed infections were identified in 23% of positive individuals. Among 453 school children with a single infection, ST2 was predominant, followed by ST1, ST3, ST7, ST10, and ST14; this is the first report of ST10 and ST14 in humans. Genetic polymorphisms were evident at the intra-ST level with the identification of numerous ST1 to ST3 genotypes. ST1 showed the greatest intra-ST diversity followed by ST2 and ST3. The prevalence and distribution of STs and genotypes varied among target villages, pointing to several potential infection sources, including human-to-human, zoonotic, and waterborne transmission.

Appendix

Article 3:

Salma Khaled, Nausicaa Gantois, Aisha Ayoubi, Gaël Even, **Manasi Sawant**, Jinane El Houmayraa, Mathieu Nabot, Sadia Benamrouz-Vanneste, Magali Chabé, Gabriela Certad, Dima El Safadi, Fouad Dabboussi, Monzer Hamze, Eric Viscogliosi (2021) *Blastocystis* sp. prevalence and subtypes distribution amongst Syrian refugee communities living in North Lebanon. *Microorganisms 9, 184*

Contribution of the student to the study

- Sequence analysis and alignment of the obtained sequences
- Interpretation of the data obtained
- Presentation of data at conference

Abstract

Molecular data concerning the prevalence and subtype (ST) distribution of the intestinal parasite *Blastocystis* sp. remain scarce in the Middle East. Accordingly, we performed the first molecular epidemiological survey ever conducted in the Syrian population. A total of 306 stool samples were collected from Syrian refugees living in 26 informal tented settlements (ITS) subjected or not to water, sanitation, and hygiene (WASH) interventions in North Lebanon, then screened for the presence of *Blastocystis* sp. by real-time polymerase chain reaction followed by subtyping. The overall prevalence of the parasite was shown to reach 63.7%. *Blastocystis* sp. colonization was not significantly associated with gender, age, symptomatic status, abdominal pain or diarrhea. In contrast, WASH intervention status of ITS was identified as a risk factor for infection. Among a total of 164 subtyped isolates, ST3 was predominant, followed by ST1, ST2, and ST10. No particular ST was reported to be associated with age, gender, symptomatic status, digestive disorders, or WASH intervention status of ITS. Intra-ST diversity of ST1 to ST3 was low suggesting large-scale anthroponotic transmission. Moreover, comparative analysis of ST1 to ST3 genotypes revealed that the circulation of the parasite between Syrian refugees and the host population was likely limited.

Appendix

Article 4:

Timothé Guilavogui, Nausicaa Gantois, Gaël Even, Jeremy Desramaut, Ellena Dautel, Constance Denoyelle, Fode Ibrahima Cissé, Salif Cherif Touré, Bakary Luther Kourouma, **Manasi Sawant**, Magali Chabé, Gabriela Certad, Eric Viscogliosi (2022) Detection, molecular identification and transmission of the intestinal protozoa *Blastocystis* sp. in Guinea from a large-scale epidemiological study conducted in the Conakry area. *Microorganisms 10, 446*

Contribution of the student to the study

- Alignment of the obtained sequences
- Interpretation of the data obtained

Abstract

Blastocystis sp. is a single-celled parasite estimated to colonize the digestive tract of 1 to 2 billion people worldwide. Although it represents the most frequent intestinal protozoa in human stools, it remains still under-investigated in countries with a high risk of infection due to poor sanitary and hygiene conditions, such as in Africa. Therefore, the present study was carried out to determine the prevalence and subtype (ST) distribution of *Blastocystis* sp. in the Guinean population. For this purpose, fecal samples were collected from 500 individuals presenting or not digestive disorders in two hospitals of Conakry. Search for the parasite in stools was performed by real-time PCR targeting the small subunit rDNA gene followed by sequencing of the PCR products for subtyping of the isolates. A total of 390 participants (78.0%) were positive for *Blastocystis* sp. Five STs were identified in the Guinean cohort (ST1, ST2, ST3, ST4 and ST14) with varying frequency, ST3 being predominant. Among them, ST4 was found in only two patients confirming its global rarity in Africa whereas infections by ST14 were likely the result of zoonotic transmission from bovid. No significant association was detected between *Blastocystis* sp. colonization or ST distribution and the symptomatic status of Guinean subjects or the presence of digestive symptoms. In contrast, drilling water consumption represented a significant risk factor for infection by *Blastocystis* sp. Predominance of ST3 coupled with its low intra-ST diversity strongly suggested large-scale human-to-human transmission of this ST within this cohort. In parallel, the highest intra-ST diversity of ST1 and ST2 was likely correlated with various potential sources of infection in addition to anthroponotic transmission. These findings highlighted the active circulation of the parasite in Guinea as reported in some low-income African countries and the necessity to implement prevention and control measures in order to limit the circulation of this parasite in this endemic geographical area.

Appendix

Article 5:

Wil JM Landman, Nausicaa Gantois, **Manasi Sawant**, FA Majoor, JHH van Eck, Eric Viscogliosi (2021) Prevalence of trichomonads in the cloaca of wild wetland birds in the Netherlands. *Avian Pathology 50, 465-476*

Contribution of the student to the study

- Construction of phylogenetic trees
- Interpretation of the data obtained
- Drafting of the article

Abstract

Severe granulomatosis in productive layer chickens due to *Tetratrichomonas gallinarum* strain 13/16632 infection occurred in 2013 and 2017 on farms situated in a wetland area in the Netherlands. We hypothesized that wetland birds could be the source of the infection. Therefore, a prevalence study on trichomonads was performed by analysing cloaca swabs of 526 birds belonging to 13 species of wetland birds. The number of birds sampled ranged from 1 to 275 per species. Birds were sampled at 15 locations in the Netherlands. DNA extracted from the cloaca swabs was subjected to nested PCR using trichomonad-specific primers targeting the internal transcribed spacer 1 (ITS1)-5.8S rRNA-ITS2 region followed by cloning and sequencing. In nine bird species, trichomonads were detected; the overall prevalence was 9% (47/526), while the prevalence in the five species for which a substantial number of birds were examined (at least 39 per species) ranged from 4% to 24%. Three trichomonad species were found: *T. gallinarum*, *Trichomonas tenax* and *Simplicimonas* sp. of which *T. gallinarum* dominated. The virulent *T. gallinarum* strain 13/16632 was not detected, but closely related strains were. Phylogenetic analysis revealed that all *T. gallinarum* isolates belonged to two clusters within lineage 15 of *Tetratrichomonas* lineages. All *T. tenax* isolates were identical and clustered with reference strain H95, while *Simplicimonas* sp. isolates showed large genetic diversity. Some isolates may represent a new species of the genus *Simplicimonas*. We conclude that trichomonads are widespread amongst wetland birds, raising the question, amongst others, of their relevance for commercial poultry.

7. Résumé plus développé de la thèse en français

Cryptosporidium est l'une des causes majeures de diarrhée chez les jeunes enfants et les patients sidéens. C'est un protozoaire parasite opportuniste appartenant au phylum des Apicomplexa qui se transmet par voie féco-orale. La maladie associée à cette infection est la cryptosporidiose. Elle touche un grand nombre de vertébrés dont l'homme et se transmet via la consommation d'eau ou d'aliments contaminés ou par contact direct avec un individu contaminé. Elle est auto-résolutive chez les individus immunocompétents mais peut être chronique voir létale chez les individus immunodéprimés. La forme infectante du parasite est l'oocyste sporulé qui une fois ingéré suit un cycle biologique monoxène. Ce dernier débute par la pénétration des sporozoïtes, libérés des oocystes, dans les cellules épithéliales puis s'en suit un cycle asexué et un cycle sexué. La réplication du parasite ainsi que la réponse immunitaire qu'il induit chez l'hôte contribuent à sa pathogénicité. Il a également été suggéré dans un grand nombre d'études épidémiologiques, une association entre la cryptosporidiose et la pathologie cancéreuse digestive. Il a ainsi été prouvé expérimentalement chez un modèle murin immunodéprimé que *Cryptosporidium parvum (C. parvum)* était doté d'un pouvoir carcinogène lui permettant d'induire des néoplasies digestives invasives. Cependant, sa pathogénicité n'est pas totalement comprise et malgré le fait qu'il soit considéré comme un véritable problème de santé public par l'OMS depuis 2006, aucun traitement efficace n'est disponible à ce jour. Pour toutes ces raisons, il semblait important de se focaliser sur l'étude des interactions entre *Cryptosporidium* et sa cellule hôte ainsi que sur le rôle de ce parasite dans le développement de cancers digestifs. Dans la première partie de ma thèse, des généralités sur le parasite sont développées ainsi que la problématique de notre étude. Cette partie s'accompagne de deux revues dont je suis premier auteur à savoir 1) « La cryptosporidiose et son impact en santé publique », Revue de biologie Médicale, 2020, 352, 53-58 et 2) «*Cryptosporidium* and colon cancer: cause or consequence?» *Microorganisms* 2020, 8, e1665.

Les objectifs principaux de ce travail de thèse ont été i) L'étude du rôle des phénomènes épigénétiques sur les interactions hôte-parasite et ii) l'exploration des voies de signalisation impliquées dans le développement des cancers digestifs induits par *C. parvum*. En effet, l'une des stratégies permettant de limiter la pathogénicité du parasite pourrait être de cibler les processus régulant l'expression génique des facteurs de virulence du parasite. En outre, l'absence chez *Cryptosporidium*, d'un nombre important de familles de facteurs de transcription pourtant présents chez les autres organismes eucaryotes suggère que le contrôle

de l'expression de ses gènes tout comme la différentiation cellulaire seraient liés à des mécanismes épigénétiques. Il semblait alors très intéressant d'explorer ces mécanismes et plus particulièrement, le rôle des phénomènes de méthylation des résidus lysine des histones sur l'expression des gènes de *C. parvum* durant l'infection.

Une analyze *in silico* a d'abord été réalisée afin d'identifier de potentielles lysine méthyltransférases des histones (HKMTs) chez *C. parvum*. Les premiers alignements de séquences ainsi que l'analyze phylogénique ont permis l'identification de différentes HKMTs du parasite et d'en prédire les substrats associés. De plus, la modélisation par homologie de ces protéines a permis de prédire trois structures de HKMTs potentiellement actives nommées CpSET1, CpSET2 et CpSET8. Leurs rôles fonctionnels ont été déterminés durant le cycle de développement du parasite grâce à l'utilisation d'anticorps spécifiques reconnaissant les résidus lysines des histones méthylées (CpSET1:H3K4; CpSET2:H3K36; CpSET8:H4K20). Afin de montrer l'importance des HKMTs dans la pathogénicité de *Cryptosporidium*, nous avons cloné et produit la CpSET8 dans un système bactérien et avons réalisé des test d'activité *in vitro* confirmant l'activité de cette HKMT. Il s'agit donc de la première étude montrant la présence de mécanismes épigénétiques chez le parasite *C. parvum* pouvant être ciblés, dans un futur proche, via le développement de drogues anti-HKMT pour lutter contre la cryptosporidiose. En parallèle, nous nous sommes également intéressés aux modifications des phénomènes de méthylation des histones de la cellule hôte infectée par *C. parvum*. En effet, il a été rapporté, dans un système de culture *in vitro*, que *C. parvum* était capable de réprimer l'expression de certains gènes de la cellule qu'il infecte. Nous avons alors entrepris d'utiliser le modèle de culture de cellules HCT8 et le modèle d'étude de la cryptosporidiose développé par les membres de l'équipe ECOPHIP, à savoir des souris SCID (Severe Combine Immunodeficiency) traitées à la dexaméthasone. Ceci nous a permis de montrer que la lésion induite par le parasite conduisait à une perte massive de la méthylation des résidus lysine des histones tels que H3K4Me3, H3K27Me3, H3K36Me3 et H4K20Me3. Le fait que H3K4Me3 soit sous exprimé pourrait réprimer l'expression de gènes de l'immunité innée et ainsi contribuer à maintenir une infection chronique. On peut donc supposer que l'une des stratégies de survie et de propagation de *C. parvum* serait l'utilisation des mécanismes épigénétiques de son hôte. Les résultats de ce travail ont été compilés dans un article dont je suis premier-auteur intitulé et soumis à la revue Virulence.

En parallèle, et toujours en utilisant le modèle de souris SCID traitées à la déxaméthasone et infectées par *C. parvum*, nous avons voulu continuer à explorer, via une approche transcriptomique cette fois-ci, les mécanismes moléculaires par lesquels ce parasite induisait le développement des néoplasies. L'idée était de réaliser une analyze comparative du profil d'expression des gènes des cellules de souris infectées *versus* non infectées par *C. parvum*. Ce travail a permis de montrer que le parasite était capable de résister à la surexpression des gènes de l'hôte stimulés par IFNγ tels que *GBPs* (Guanylate Binding Proteins) et *IIGP1* (Interferon inducible GTPase 1) afin de persister chez son hôte. Le mécanisme lui conférant cette résistance demeure inconnu. Toutefois, il semblerait que la réaction inflammatoire induite par l'infection à *C. parvum* conduirait à une régulation négative de gènes codant des molécules antimicrobiennes telle que les α-défensines (*DEFA1* et *DEFA4*). Il est donc possible que nous ayons réussi à identifier une nouvelle cible de *C. parvum* qui pourrait contribuer à sa stratégie d'échappement à la réponse immunitaire de l'hôte et donc permettre la persistance de l'infection. De plus, la diminution de l'expression des gènes codant les α-défensines dans les tissus transformés suite à l'infection à *C. parvum*, met en exergue le rôle de l'inflammation dans l'induction du cancer par ce parasite. Ces résultats ont été compilés dans un article pour lequel je suis premier auteur intitulé «Persistent *Cryptosporidium parvum* infection leads to the development of the tumor microenvironment in an experimental mouse model: results of a microarray approach. *Microorganisms* 2021, 9, e2569. ».

Toujours dans l'optique d'élucider les mécanismes de carcinogénèse induits pas l'infection à *C. parvum,* et à la lumière des résultats obtenus à la suite de l'analyze transcriptomique, nous nous sommes aussi intéressés plus particulièrement à la voie phosphatidylinositol 3-kinase (PI3K)/AKT. En effet, cette voie semble, elle aussi, être modulée et serait potentiellement impliquée également dans la réorganisation du cytosquelette de la cellule hôte lors de l'infection à *C. parvum*. Or, ces altérations du cytosquelette semblent être un processus majeur lors de l'induction des néoplasies par le parasite. Pour ce faire, nous avons entrepris de réaliser des analyzes d'enrichissement des voies (pathway enrichment analysis) ainsi que des analyzes biochimiques (Western blot) et immunohistochimiques sur des tissus coliques infectées ou non par *C. parvum* issus de souris SCID. Les résultats obtenus montrent clairement une augmentation de l'expression aussi bien de la PI3K que de la forme phosphorylée de l'AKT suggérant une activation de cette voie lors du processus de transformation cellulaire induits par l'infection. Cependant, d'autres expérimentations notamment d'inhibition des acteurs de la voie PI3K/AKT sont nécessaires pour confirmer leurs

rôles dans ce processus. Cette voie mérite une attention particulière car au-delà des raisons évoquées ci-dessus, il a été montré qu'elle régulait négativement les méthylations H3K27 et H3K4 ce qui a comme conséquence l'induction du phénomène de transition épithélio-mésenchymateuse (TEM) dans les cas de cancers gastriques. Or, comme indiqué précédemment, nous avons montré que l'infection induisait une régulation négative de ces mêmes méthylations dans les zones de néoplasies induites par *C. parvum*. Ceci met en évidence une probable capacité du parasite à induire des transformations cellulaires via la régulation de gènes responsables de la TEM. L'hypothèse selon laquelle la TEM se produirait dans le microenvironnement tumoral induit par le parasite est confortée par le fait que différents gènes codant diverse métalloprotéines (*MMPs*) et sérine protéases (*PLAU*) connues pour être associées au remodelage de la matrice extracellulaire ont également été identifiés lors de l'analyze transcriptomique. Les résultats de cette recherche ont été compilés dans un manuscrit en préparation intitulé « The role of PI3K/AKT signaling pathway in *C. parvum*-induced digestive neoplasia ».

Le travail réalisé dans le cadre de cette thèse a donc permis de confirmer (i) que le parasite peut détourner les voies de signalisation cellulaires de l'hôte pour favoriser un environnement propice à la multiplication des parasites mais également (ii) que l'altération de certaines de ces voies a pour conséquence la prolifération cellulaire, l'inhibition de l'apoptose et l'échappement à la réponse immunitaire. L'étude du mécanisme épigénétique à savoir la méthylation des résidus lysine des histones a quant à elle permis de fournir les premières données concernant la régulation de l'expression de gènes de *C. parvum* durant son cycle de vie. L'approche épigénétique combinée à l'approche transcriptomique nous ont permis de mettre en évidence que le parasite est capable de détourner et d'altérer les mécanismes cellulaires de l'hôte ce qui conduit au développement de cancer. En perspectives, l'ensemble de ces travaux ouvre de nouvelles voies pour accentuer la recherche sur *C. parvum* et ses interactions avec son hôte. Cela aura pour conséquence de mieux contrôler l'infection et ses impacts sur la santé humaine et animale et éventuellement, sur le développement de cancers digestifs.

Pour terminer et dans le but d'élargir mes connaissances et compétences dans le domaine de la parasitologie, j'ai eu l'opportunité au cours de ma thèse d'être impliquée dans trois autres projets de recherche non développés dans mon manuscrit de thèse mais listés en Annexes. Le premier portait sur l'épidémiologie de *Cryptosporidium* dans les poissons marins comestibles avec l'identification moléculaire de nouveaux génotypes (*Microorganisms* 2020,

8, 2014). Le second portait sur une autre thématique majeure de l'équipe, à savoir, l'étude de la prévalence et de la circulation du protozoaire intestinal *Blastocystis* dans la population humaine à travers de larges enquêtes épidémiologiques menées au Sénégal (*Microorganisms* 2020, 8, 1408), dans les camps de réfugiés syriens au Nord Liban (*Microorganisms* 2021, 9, 184) et plus récemment en Guinée (*Microorganisms* 2022, 10, 446). Le dernier projet portait sur la prévalence et l'identification moléculaire de protozoaires appartenant au groupe des trichomonadines chez les oiseaux sauvages des zones humides aux Pays-Bas, en étroite collaboration avec Royal GD, une organisation néerlandaise de premier plan dans le domaine de la santé animale (*Avian Pathology* 2021, 50, 465-476).

8. References

- Abdou, Asmaa Gaber, Nancy Mahmoud Harba, Amira Fathy Afifi, and Nada Farag Elnaidany. 2013. "Assessment of *Cryptosporidium parvum* Infection in Immunocompetent and Immunocompromised Mice and Its Role in Triggering Intestinal Dysplasia." *International Journal of Infectious Diseases* 17(8):e593–600. doi: 10.1016/j.ijid.2012.11.023.
- Abrahamsen MS, Templeton TJ, Enomoto S, Abrahante JE, Zhu G, Lancto CA, Deng M, Liu C, Widmer G, and Tzipori S. 2004. "Complete Genome Sequence of the Apicomplexan, Cryptosporidium Parvum." *Science* 304(5669):441–45. doi: 10.1126/science.1094786.
- Allfrey VG, Faulkner R, and Mirsky AE. 1964. "Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis." *Proc Natl Acad Sci U S A* 51:786–94. doi: 10.1073/pnas.51.5.786.
- Arisue N and Hashimoto T. 2015. "Phylogeny and Evolution of Apicoplasts and Apicomplexan Parasites." *Parasitol Int* 64:254–59. doi: 10.1016/j.parint.2014.10.005.
- Audebert, Christophe, Franck Bonardi, Ségolène Caboche, Karine Guyot, Hélène Touzet, Sophie Merlin, Nausicaa Gantois, Colette Creusy, Dionigia Meloni, Anthony Mouray, Eric Viscogliosi, Gabriela Certad, Sadia Benamrouz-Vanneste, and Magali Chabé. 2020. "Genetic Basis for Virulence Differences of Various *Cryptosporidium parvum* Carcinogenic Isolates." *Scientific Reports* 10(1). doi: 10.1038/s41598-020-64370-0.
- Baldursson S and Karanis P. 2011. "Waterborne Transmission of Protozoan Parasites: Review of Worldwide Outbreaks—An Update 2004–2010." *Water Res* 45:6603–14.
- Bannister A.J, Schneider R, and Kouzarides T. 2002. "Histone Methylation: Dynamic or Static?" *Cell* 109:801–6. doi: 10.1016/S0092-8674(02)00798-5.
- Bañuls, Anne-Laure, Frédéric Thomas, and François Renaud. 2013. "Of Parasites and Men." *Infection, Genetics and Evolution : Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases* 20:61–70. doi: 10.1016/j.meegid.2013.08.005.
- Barnes CE, English DM, and Cowley SM. 2019. "Acetylation & Co: An Expanding Repertoire of Histone Acylations Regulates Chromatin and Transcription." *Essays Biochem* 63(1):97–107. doi: 10.1042/EBC20180061.
- Baydoun M, Benamrouz-Vanneste S, Cruesy C, Guyot K, Gantois N, Chabé M, Delaire B, Mouray A, Baydoun A, and Forzy G. 2017. "Three-Dimensional (3D)

References

- Culture of Adult Murine Colon as an in Vitro Model of Cryptosporidiosis: Proof of Concept." *Sci Rep* 7:17288. doi: 10.1038/s41598-017-17304-2.
- Benamrouz S, Conseil V, Chabé M, Praet M, Audebert C, Blervaque R, Guyot K, Gazzola S, Mouray A, and Chassat T. 2014. "Cryptosporidium Parvum-Induced Ileo-Caecal Adenocarcinoma and Wnt Signaling in a Mouse Model." *Dis Model Mech* 7:693–700. doi: 10.1242/dmm.013292.
- Benamrouz, Sadia, Valerie Conseil, Magali Chabé, Marleen Praet, Christophe Audebert, Renaud Blervaque, Karine Guyot, Sophie Gazzola, Anthony Mouray, Thierry Chassat, Baptiste Delaire, Nathalie Goetinck, Nausicaa Gantois, Marwan Osman, Christian Slomianny, Vanessa Dehennaut, Tony Lefebvre, Eric Viscogliosi, Claude Cuvelier, Eduardo Dei-Cas, Colette Creusy, and Gabriela Certad. 2014. "Cryptosporidium Parvum-Induced Ileo-Caecal Adenocarcinoma and Wnt Signaling in a Mouse Model." *Disease Models & Mechanisms* 7(6):693–700. doi: 10.1242/dmm.013292.
- Benamrouz, Sadia, Valérie Conseil, Colette Creusy, Enrique Calderon, Eduardo Dei-Cas, and Gabriela Certad. 2012. "Parasites and Malignancies, a Review, with Emphasis on Digestive Cancer Induced by Cryptosporidium parvum(Alveolata: Apicomplexa)." *Parasite (Paris, France)* 19(2):101–15.
- Biggar K. K and Shawn S.-C. Li. 2015. "Non-Histone Protein Methylation as a Regulator of Cellular Signaling and Function." *Nat Rev Mol Cell Biol* 16:5–17. doi: 10.1038/nrm3915.
- Borad A and Ward H. 2010. "Human Immune Responses in Cryptosporidiosis." *Future Microbiol* 5:507–19. doi: 10.2217/fmb.09.128.
- Bouvard, Véronique, Robert Baan, Kurt Straif, Yann Grosse, Béatrice Secretan, Fatiha El Ghissassi, Lamia Benbrahim-Tallaa, Neela Guha, Crystal Freeman, Laurent Galichet, Vincent Cogliano, and on behalf of the WHO International Agency for Research on Cancer Monograph Working Group. n.d. *Special Report: Policy A Review of Human Carcinogens-Part B: Biological Agents*. Vol. 10. doi: 10.1016/S1470-2045(09)70096-8.
- Braun L, Brenier-Pinchart MP, Hammoudi PM, Cannella D, Kieffer-Jaquinod S, Vollaire J, Josserand V, Touquet B, Coute Y, Tardieux I, Bougdour A, and Hakimi MA. 2019. "The *Toxoplasma* Effector TEEGR Promotes Parasite Persistence by Modulating NF-KB Signaling via EZH2." *Nat Microbiol* 4:1208–20.
- Brooks CF, Francia ME, Gissot M, Croken MM, KIM K, and Streipen B. 2011. "*Toxoplasma gondii* Sequesters Centromeres to a Specific Nuclear Region throughout the Cell Cycle." *Proc Natl Acad Sci U S A* 108:3767–72. doi: 10.1073/pnas.1006741108.
- Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, and Allis CD. 1996. "Tetrahymena Histone Acetyltransferase A: A Homolog to Yeast Gcn5p Linking Histone Acetylation to Gene Activation." *Cell* 84:843–51. doi: 10.1016/s0092-8674(00)81063-6.

References

- Certad G, Creusy C, Guyot K, Mouray A, Chassat T, Delaire B, Pinon A, Sitja-Bobadilla A, Alvarez-Pellitero P, and Praet M. 2010. “Fulminant Cryptosporidiosis Associated with Digestive Adenocarcinoma in SCID Mice Infected with Cryptosporidium parvumTUM1 Strain.” *Int J Parasitol* . 40:1469–75. doi: 10.1016/j.ijpara.2010.07.007.
- Certad G, Cruesy C, Ngouanesavanh T, Guyot K, Gantois N, Mouray A, Chassat T, Flament N, Fleurisse L, Pinon A, Delhaes L, and Dei-Cas E. 2010. “Development of Cryptosporidium Parvum-Induced Gastrointestinal Neoplasia in Severe Combined Immunodeficiency (SCID) Mice: Severity of Lesions Is Correlated with Infection Intensity.” *Am J Trop Med Hyg* 82:257-265. doi: 10.4269/ajtmh.2010.09-0309.
- Certad G, Follet J, Gantois N, Hammouma-Ghelboun O, Guyot K, Benamrouz-Vanneste S, Fréalle E, Seesao Y, Delaire B, and Creusy C. 2019. “Prevalence, Molecular Identification, and Risk Factors for Cryptosporidium Infection in Edible Marine Fish: A Survey Across Sea Areas Surrounding France.” *Front. Microbiol* 10:1037. doi: 10.3389/fmicb.2019.01037.
- Certad G, Ngouanesavanh T, Guyot K, Gantois N, Chassat T, Mouray A, Fleurisse L, Pinon A, Cailliez JC, and Dei-Cas, E. 2007. “Cryptosporidium Parvum, a Potential Cause of Colic Adenocarcinoma.” *Infect. Agents Cancer* 2:22.
- Certad G, Zahedi A, Gantois N, Sawant M, Creusy C, Duval E, Benamrouz-Vanneste S, Ryan U, and Viscogliosi E. 2020. “Molecular Characterization of Novel Cryptosporidium Fish Genotypes in Edible Marine Fish.” *Microorganisms* 8:2014. doi: 10.3390/microorganisms8122014.
- Certad, Gabriela, Sadia Benamrouz, Karine Guyot, Anthony Mouray, Thierry Chassat, Nicolas Flament, Laurence Delhaes, Valerie Coiteux, Baptiste Delaire, Marleen Praet, Claude Cuvelier, Pierre Gosset, Eduardo Dei-Cas, and Colette Creusy. 2012. “Fulminant Cryptosporidiosis after Near-Drowning: A Human *Cryptosporidium parvum* Strain Implicated in Invasive Gastrointestinal Adenocarcinoma and Cholangiocarcinoma in an Experimental Model.” *Applied and Environmental Microbiology* 78(6):1746–51.
- Certad, Gabriela, Colette Creusy, Karine Guyot, Anthony Mouray, Thierry Chassat, Baptiste Delaire, Anthony Pinon, Ariadna Sitjà-Bobadilla, Pilar Alvarez-Pellitero, Marleen Praet, Claude Cuvelier, and Eduardo Dei-Cas. 2010. “Fulminant Cryptosporidiosis Associated with Digestive Adenocarcinoma in SCID Mice Infected with *Cryptosporidium parvum* TUM1 Strain.” *International Journal for Parasitology* 40(13):1469–75.
- Chalmers RM, Robertson LJ, Dorny P, Jordan S, Kärssin A, Katzer F, La Carbona S, Lalle M, Lassen B, and Mladineo I. 2020. “Parasite Detection in Food: Current Status and Future Needs for Validation.” *Trends Food Sci. Technol* 99:337–50. doi: 10.1016/j.tifs.2020.03.011.
- Checkley W, White Jr AC, Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, Fayer R, Griffiths JK, Guerrant RL, and Hedstorm L. 2015. “A Review of the Global

References

Burden, Novel Diagnostics, Therapeutics, and Vaccine Targets for Cryptosporidium." *Lancet Infect Dis.* 15(1):85–94.

- Cheeseman, K, Jannot, G, Lourenço, N, Villares M, Berthelet J, Calegari-Silva T, Hamroune J, Letourneur F, Rodrigues-Lima F, and Weitzman JB. 2021. "Dynamic Methylation of Histone H3K18 in Differentiating *Theileria* Parasites." *Nat Commun* 12(3221). doi: 10.1038/s41467-021-23477-2.

- Cheeseman, Kevin, Gabriela Certad, and Jonathan B. Weitzman. 2016. "Parasites et Cancer : Existe-t-Il Un Lien ?" *Médecine/Sciences* 32(10):867–73. doi: 10.1051/medsci/20163210020.

- Chen VB, Arendall WB, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, and Richardson DC. 2009. "MolProbity: All-Atom Structure Validation for Macromolecular Crystallography." *Acta Crystallogr D Biol Crystallogr* 66:12–21. doi: 10.1107/S0907444909042073.

- Chen, Xian Ming, Patrick L. Splinter, Pamela S. Tietz, Bing Q. Huang, Daniel D. Billadeau, and Nicholas F. LaRusso. 2004. "Phosphatidylinositol 3-Kinase and Frabin Mediate *Cryptosporidium parvum* Cellular Invasion via Activation of Cdc42." *The Journal of Biological Chemistry* 279(30):31671–78. doi: 10.1074/JBC.M401592200.

- Cheng X, Collins RE, and Zhang X. 2005. "Structural and Sequence Motifs of Protein (Histone) Methylation Enzymes." *Annu Rev Biophys Biomol Struct.* 34:267-294. doi: 10.1146/annurev.biophys.34.040204.144452.

- Chookajorn T, Dzikowski R, Frank M, Li F, Jiwani AZ, Hartl DL, and Dietsch KW. 2007. "Epigenetic Memory at Malaria Virulence Genes." *Proc Natl Acad Sci U S A* 104(3):899–902. doi: 10.1073/pnas.0609084103.

- Choudhry N, Korbel DS, Edwards LA, Bajaj-Elliott M, and McDonald V. 2009. "Dysregulation of Interferon-Gamma-Mediated Signaling Pathway in Intestinal Epithelial Cells by *Cryptosporidium parvum* Infection." *Cell. Microbiol* 11:1354–64. doi: 10.1111/j.1462-5822.2009.01336.

- Chua MJ, Arnold MSJ, Xu W, Lancelot J, Lamotte S, Spath GF, Prina E, Pierce RJ, Fairlie DP, Skinner-Adams TS, and Andrews KT. 2017. "Effect of Clinically Approved HDAC Inhibitors on *Plasmodium*, Leishmania and Schistosoma Parasite Growth." *Int J Parasitol Drugs Drug Resist.* 7(1):42-50. doi: 10.1016/j.ijpddr.2016.12.005.

- Cock-Rada AM, Medjkane S, Janski N, Yousfi N, Perichon M, Chaussepied M, Chluba J, Langsley G, and Weitzman JB. 2012. "SMYD3 Promotes Cancer Invasion by Epigenetic Upregulation of the Metalloproteinase MMP-9." *Cancer Res* 72:810–20.

- Cui L, Fan Q, Cui L, and Miao J. 2008. "Histone Lysine Methyltransferases and Demethylases in *Plasmodium falciparum*." *Int J Parasitol.* 38:1083-1097. doi: 10.1016/j.ijpara.2008.01.002.

References

- Currey N, Jahan Z, Caldon CE, Tran PN, Benthani F, De Lacavalerie P, Roden DL, Gloss BS, Campos C, and Bean EG. 2019. “Mouse Model of Mutated in Colorectal Cancer Gene Deletion Reveals Novel Pathways in Inflammation and Cancer.” *Cell Mol Gastroenterol Hepatol* 7:819–39. doi: 10.1016/j.jcmgh.2019.01.009.
- Darkin-Rattray S.J, Gurnett AM, Myers RW, Dulski PM, Crumley TM, Allocco JJ, Cannova C, Meinke PT, Colletti SL, and Bednarek MA. 1996. “Apicidin: A Novel Antiprotozoal Agent That Inhibits Parasite Histone Deacetylase.” *Proc. Natl. Acad. Sci* 93:13143–47. doi: 10.1073/pnas.93.23.13143.
- Dibao-Dina A, Follet J, Ibrahim M, Vlandas A, and Senez V. 2015. “Electrical Impedance Sensor for Quantitative Monitoring of Infection Processes on HCT-8 Cells by the Waterborne Parasite *Cryptosporidium*.” *Biosens. Bioelectron* 66:69–76. doi: 10.1016/j.bios.2014.11.009.
- Dillon SC, Zhang X, Trievel RC, and Cheng X. 2005. “The SET-Domain Protein Superfamily: Protein Lysine Methyltransferases.” *Genome Biol* 6:227. doi: 10.1186/gb-2005-6-8-227.
- Dixon SE, Stilger KL, Elias EV, Naguleswaran A, and Sullivan WJ., Jr. 2010. “A Decade of Epigenetic Research in *Toxoplasma gondii*.” *Mol Biochem Parasitol.* 173:1–9. doi: 10.1016/j.molbiopara.2010.05.001.
- Duffy MF, Selvahraj SA, Josling GA, and Petter M. 2014. “Epigenetic Regulation of *Plasmodium falciparum* Genome.” *Brief Funct Genomics* 13:203–16. doi: 10.1093/bfgp/elt047.
- Duijster JW, Franz E, Neefjes J, and Mughini-Gras L. 2021. “Bacterial and Parasitic Pathogens as Risk Factors for Cancers in the Gastrointestinal Tract: A Review of Current Epidemiological Knowledge.” *Front Microbiol* 12:790256. doi: 10.3389/fmicb.2021.790256.
- Dumaine JE, Tandel J, and Striepen B. 2019. “*Cryptosporidium parvum*.” *Trends Parasito*. doi: 10.1016/j.pt.2019.11.003.
- Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, and DuBois RN. 1994. “Up-Regulation of Cyclooxygenase 2 Gene Expression in Human Colorectal Adenomas and Adenocarcinomas.” *Gastroenterology* 107:1183–88. doi: 10.1016/0016-5085(94)90246-1.
- Edmunds JW, Mahadevan LC, and Clayton AL. n.d. “Dynamic Histone H3 Methylation during Gene Induction: HYPB/Setd2 Mediates All H3K36 Trimethylation.” *EMBO J* 27:406–20. doi: 10.1038/sj.emboj.7601967.
- Efstratiou A, Ongerth JE, and Karanis P. 2017. “Waterborne Transmission of Protozoan Parasites: Review of Worldwide Outbreaks—An Update 2011–2016.” *Water Res* 114:14–22.
- Egorova KS, Olenkina OM, and Olenina LV. 2010. “Lysine Methylation of Nonhistone Proteins Is a Way to Regulate Their Stability and Function.” *Biochemistry (Mosc)* . 75:535–48. doi: 0.1134/s0006297910050019.

References

- English ED, Guérin A, Tandel J, and Striepen B. 2021. "Live Imaging of the *Cryptosporidium parvum* Lifecycle Reveals Direct Development of Male and Female Gametes from Type One Meronts." *Pre-Print*. doi: 10.1101/2021.12.07.471643.
- Ewald PW. 2009. "An Evolutionary Perspective on Parasitism as a Cause of Cancer." *Adv Parasitol* 68:21–43. doi: 10.1016/S0065-308X(08)00602-7.
- Fan Q, An L, and Cui L. 2004. "*Plasmodium falciparum* Histone Acetyltransferase, a Yeast GCN5 Homologue Involved in Chromatin Remodeling." *Eukaryot Cell* 3:264–76. doi: 10.1128/EC.3.2.264-276.2004.
- Feng Y, Ryan U, and (last) Xiao L. 2018. "Genetic Diversity and Population Structure of Cryptosporidium." *Trends Parasito* 34(11):997–1107.
- Foltz C, Napolitano A, Khan R, Clough B, Hirst EM, and Frickel EM. 2017. "TRIM21 Is Critical for Survival of *Toxoplasma gondii* Infection and Localizes to GBP-Positive Parasite Vacuoles." *Sci. Rep* 7:5209. doi: 10.1038/s41598-017-05487-7.
- Forney JR, Dewald DB, Yang S, Speer CA, and Healey MC. 1999. "A Role for Host Phosphoinositide 3-Kinase and Cytoskeletal Remodeling during Cryptosporidium parvumInfection." *Infect Immun* 67:844–52. doi: 10.1128/IAI.67.2.844-852.1999.
- Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, and Bowman S. 2002. "Genome Sequence of the Human Malaria Parasite *Plasmodium falciparum*." *Nature* 3:498–511. doi: 10.1038/nature01097.
- Gillrie MR, Lee K, Gowda DC, Davis SP, Monestier M, Cui L, Hien TT, Day NPJ, and Ho M. 2012. "*Plasmodium falciparum* Histones Induce Endothelial Proinflammatory Response and Barrier Dysfunction." *Am J Pathol* 180:1028–39. doi: 10.1016/j.ajpath.2011.11.037.
- Gissot M, Choi SW, Thompson RF, Greally JM, and Kim K. 2008. "*Toxoplasma* Gondii and *Cryptosporidium parvum* Lack Detectable DNA Cytosine Methylation." *Eukaryot Cell.* 7(3):537–40. doi: 10.1128/EC.00448-07.
- Gissot M and KIM K. 2008. "How Epigenomics Contributes to the Understanding of Gene Regulation in *Toxoplasma gondii.*" *J Eukaryot Microbiol* 55:476-480. doi: 10.1111/j.1550-7408.2008.00366.x.
- Greer EL and Shi Y. 2012. "Histone Methylation: A Dyanamic Mark in Health, Disease and Inheritance." *Nat Rev Genet* 13:343–57. doi: 10.1038/nrg3173.
- Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, and Lee-Kwon W. 2003. "The Protein Kinase AKT Induces Epithelial Mesenchymal Transition and Promotes Enhanced Motility and Invasiveness of Squamous Cell Carcinoma Lines." *Cancer Res.* 63.
- Guesdon W, Auray G, Pezier T, Bussiere F, Drouet F, Vern YL, Marquis M, Potiron L, Rabot S, and Bruneau A. 2015. "CCL20 Displays Antimicrobial Activity against *Cryptosporidium parvum*, but Its Expression Is Reduced during Infection in the Intestine of Neonatal Mice." *J Infect Dis* 212:1332–40. doi: 10.1093/infdis/jiv206.

References

- Gullicksrud JA, Sateriale A, Engiles JB, Gibson AR, Shaw S, Hutchins ZA, Martin L, Christian DA, Taylor GA, and Yamamoto M. 2022. “Enterocyte–Innate Lymphoid Cell Crosstalk Drives Early IFN-γ-Mediated Control of *Cryptosporidium*.” *Mucosal Immunol* 15:362–72. doi: 10.1038/s41385-021-00468-6.
- Guo F, Zhang H, McNair NN, Mead JR, and Zhu G. 2018a. “The Existing Drug Vorinostat as a New Lead Against Cryptosporidiosis by Targeting the Parasite Histone Deacetylases.” *J Infect Dis* 217(7):1110–17. doi: 10.1093/infdis/jix689.
- Guo F, Zhang H, McNair NN, Mead JR, and Zhu G. 2018b. “The Existing Drug Vorinostat as a New Lead Against Cryptosporidiosis by Targeting the Parasite Histone Deacetylases.” *J Infect Dis* 217:1110-1117. doi: 10.1093/infdis/jix689.
- Hacker KE, Fahey CC, Shinsky SA, Chiang YCJ, DiFiore JV, Jha DK, Vo AH, Shavit JA, Davis IJ, and Strahl BD. 2016. “Structure/Function Analysis of Recurrent Mutations in SETD2 Protein Reveals a Critical and Conserved Role for a SET Domain Residue in Maintaining Protein Stability and Histone H3 Lys-36 Trimethylation.” *J Biol Chem.* 291:21283–95. doi: 10.1074/jbc.M116.739375.
- Hakimi MA and Deitsch KW. 2007. “Epigenetics in Apicomplexa: Control of Gene Expression during Cell Cycle Progression, Differentiation and Antigenic Variation.” *Curr Opin Microbiol.* 10(4):357–62. doi: 10.1016/j.mib.2007.07.005.
- Hakimi MA and Dietsch KW. 2007. “Epigenetics in Apicomplexa: Control of Gene Expression during Cell Cycle Progression, Differentiation and Antigenic Variation.” *Curr Opin Microbiol .* 10:357–62. doi: 10.1016/j.mib.2007.07.005.
- Hamamoto R, Furukawa Y, Morita M, Iimura Y, Silva FP, Li M, Yagyu R, and Nakamura Y. 2004. “SMYD3 Encodes a Histone Methyltransferase Involved in the Proliferation of Cancer Cells.” *Nat Cell Bio* 6:731–40. doi: 10.1038/ncb1151.
- Heaslip AT, Nishi M, Stein B, and Hu K. 2011. “The Motility of a Human Parasite, *Toxoplasma gondii,* Is Regulated by a Novel Lysine Methyltransferase.” *PLoS Pathog* 7:e1002201. doi: 10.1371/journal.ppat.1002201.
- Hettmann C and Soldati D. 1999. “Cloning and Analysis of a *Toxoplasma gondii* Histone Acetyltransferase: A Novel Chromatin Remodelling Factor in Apicomplexan Parasites.” *Nucleic Acids Res.* 27:4344-4352. doi: 10.1093/nar/27.22.4344.
- Holm L. 2020. “Using Dali for Protein Structure Comparison.” *Methods Mol Biol* 2112:29–42. doi: 10.1007/978-1-0716-0270-6_3.
- Houtz RL, Poneleit L, Jones SB, Royer M, and Stults JT. 1992. “Posttranslational Modifications in the Amino- Terminal Region of the Large Subunit of Ribulose- 1,5-Bisphosphate Carboxylase/Oxygenase from Several Plant Species.” *Plant Physiol* 98:1170–74. doi: 10.1104/pp.98.3.1170.
- Husmann D and Gozani O. 2019. “Histone Lysine Methyltransferases in Biology and Disease.” *Nat. Struct. Mol. Biol.* 26:26, 880–89.

References

- IARC Working Groupe on the Evaluation of Carcinogenic Risks to Humans. 2012. *Biological Agents, Volume 100 B, a Review of Human Carcinogens.* Lyon, France.
- Iyer LM, Anantharaman V, Wolf MY, and Aravind L. 2008. "Comparative Genomics of Transcription Factors and Chromatin Proteins in Parasitic Protists and Other Eukaryotes." *Int J Parasitol* 38:1–31. doi: 10.1016/j.ijpara.2007.07.018.
- Jain K, Fraser CS, Marunde MR, Parker MM, Sagum C, Burg JM, Hall N, Popova IR, Rodriquez KL, and Vaidya A. 2020. "Characterization of the Plasnt Homeodomian (PHD) Reader Family for Their Hisotne Tails Interactions." *Epigenetics Chromatin* 13:3. doi: 10.1186/s13072-020-0328-z.
- Jenuwein T, Laible G, Dorn R, and Reuter G. 1998. "SET Domain Proteins Modulate Chromatin Domains in Eu- and Heterochromatin." *Cell Mol Life Sci* 54:80–93. doi: 10.1007/s000180050127.
- Jiang L, Mu J, Zhang Q, Ni T, Srinivasan P, Rayavara K, Yang W, Turner L, Lavstsen T, and Theander TG. 2013. "PfSETvs Methylation of Histone H3K36 Represses Virulence Genes in *Plasmodium falciparum*." *Nature* 499:223–27. doi: 10.1038/nature12361.
- Keegan AR, Fanok S, Monis PT, and Saint CP. 2003. "Cell Culture-Taqman PCR Assay for Evaluation of *Cryptosporidium parvum* Disinfection." *Appl Environ Microbiol* 69:2505–11. doi: 10.1128/AEM.69.5.2505-2511.2003.
- Khalil IA, Troeger C, Rao PC, Blacker BF, Brown A, Brewer TG, Colombara DV, De Hostos EL, Engmann C, and Guerrant RL. 2018. "Morbidity, Mortality, and Long-Term Consequences Associated with Diarrhoea from *Cryptosporidium* Infection in Children Younger Than 5 Years: A Meta-Analyzes Study." *Lancet Glob. Healt* 6:e758–68. doi: https://doi.org/10.1016/s2214-109x(18)30283-3.
- Khurana, S., M. L. Dubey, and N. Malla. 2005. "Association of Parasitic Infections and Cancers." *Indian Journal of Medical Microbiology* 23(2):74–79.
- Khurana, S., ML Dubey, and N. Malla. 2005. "Association of Parasitic Infections and Cancers." *Indian Journal of Medical Microbiology* 23(2):74.
- Kissinger JC, Gajria B, Li L, Paulsen IT, and Roos DS. 2003. "ToxoDB: Accessing the *Toxoplasma gondii* Genome." *Nucleic Acids Res* . 1:234–36. doi: 10.1093/nar/gkg072.
- Korich DG, Mead JR, Madore MS, Sinclair NA, and Sterling CR. 1990. "Effects of Ozone, Chlorine Dioxide, Chlorine, and Monochloramine on *Cryptosporidium parvum* Oocyst Viability." *Appl Environ Microbiol* 56:1423–28. doi: 10.1128/aem.56.5.1423-1428.1990.
- Kotloff, Karen L., James P. Nataro, William C. Blackwelder, Dilruba Nasrin, Tamer H. Farag, Sandra Panchalingam, Yukun Wu, Samba O. Sow, Dipika Sur, Robert F. Breiman, Abu SG Faruque, Anita KM Zaidi, Debasish Saha, Pedro L. Alonso, Boubou Tamboura, Doh Sanogo, Uma Onwuchekwa, Byomkesh Manna, Thandavarayan Ramamurthy, Suman Kanungo, John B. Ochieng, Richard Omore,

References

Joseph O. Oundo, Anowar Hossain, Sumon K. Das, Shahnawaz Ahmed, Shahida Qureshi, Farheen Quadri, Richard A. Adegbola, Martin Antonio, M. Jahangir Hossain, Adebayo Akinsola, Inacio Mandomando, Tacilta Nhampossa, Sozinho Acácio, Kousick Biswas, Ciara E. O'Reilly, Eric D. Mintz, Lynette Y. Berkeley, Khitam Muhsen, Halvor Sommerfelt, Roy M. Robins-Browne, and Myron M. Levine. 2013. "Burden and Aetiology of Diarrhoeal Disease in Infants and Young Children in Developing Countries (the Global Enteric Multicenter Study, GEMS): A Prospective, Case-Control Study." *The Lancet* 382(9888):209–22. doi: 10.1016/S0140-6736(13)60844-2.

- Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, and Breiman RF. 2013. "Burden and Aetiology of Diarrhoeal Disease in Infants and Young Children in Developing Countries (The Global Enteric Multicenter Study, GEMS): A Prospective, Case-Control Study." *Lancet* 382:209–22. doi: https://doi.org/10.1016/s0140-6736(13)60844-2.

- Koveitypour, Zahra, Farnoush Panahi, Mehrdad Vakilian, Maryam Peymani, Farzad Seyed Forootan, Mohammad Hossein Nasr Esfahani, and Kamran Ghaedi. 2019. "Signaling Pathways Involved in Colorectal Cancer Progression." *Cell and Bioscience* 9(1):1–14. doi: 10.1186/S13578-019-0361-4/TABLES/2.

- Kumar S, Chinnusamy V, and Mohapatra T. 2018. "Epigenetics of Modified DNA Bases: 5-Methylcytosine and Beyond." *Front. Genet.* 18(9):640. doi: 10.3389/fgene.2018.00640.

- Kumar S, Stecher G, Li M, Knyaz C, and Tamura K. 2018. "MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms." *Mol Biol Evol* 35:1547–49. doi: 10.1093/molbev/msy096.

- Kunizaki M, Hamamoto R, Silva FP, Yamaguchi K, Nagayasu T, and Shibuya M. 2007. "The Lysine 831 of Vascular Endothelial Growth Factor Receptor 1 Is a Novel Target of Methylation by SMYD3." *Cancer Res* 67:10759–65. doi: 10.1158/0008-5472.CAN-07-1132.

- Laurent F and Lacroix-Lamandé S. 2017. "Innate Immune Responses Play a Key Role in Controlling Infection of the Intestinal Epithelium by *Cryptosporidium*." *Int J Parasitol* 47:711–21. doi: 10.1016/j.ijpara.2017.08.001.

- Lecoeur H, Prina E, Rosazza T, Kokou K, N'Diaye P, Aulner N, Varet H, Bussotti G, Xing Y, Milon G, Weil R, Meng G, and Spath GF. 2020. "Targeting Marcophage Histone H3 Modificaiton as a Leishmania Strategy to Dampen the NF-KB/NLRP3-Mediated Inflammatory Response." *Cell Rep* 30:1870-1882.e4. doi: 10.1016/j.celrep.2020.01.030.

- Levine MM, Nasrin D, Acácio S, Bassat Q, Powell H, Tenant SM, Sow SO, Sur D, Zaidi AKM, and Faruque ABG. 2019. "Diarrhoeal Disease and Subsequent Risk of Death in Infants and Children Residing in Low-Income and Middle-Income Countries: Analysis of the GEMS Case-Control Study and 12-Month GEMS-1A Follow-on Study." *Lancet Glob Health* . 8:E204–14. doi: 10.1016/S2214-109X(19)30541-8.

References

- Li J, Moazed D, and Gygi SP. 2002. "Association of the Histone Methyltransferase Set2 with RNA Polymerase II Plays a Role in Transcription Elongation." *J Biol Chem* 277:49383–88. doi: 10.1074/jbc.M209294200.
- Lippuner C, Ramakrishnan C, Basso WU, Schmid MW, Okoniewski M, Smith NC, Hassig M, Deplazes P, and Hehl AB. 2018. "RNA-Seq Analysis during the Life Cycle of *Cryptosporidium parvum* Reveals Significant Differential Gene Expression between Proliferating Stages in the Intestine and Infectious Sporozoites." *Int J Parasitol.* 48(6):413–22. doi: 10.1016/j.ijpara.2017.10.007.
- Liu X, Li Z, Song Y, Wang R, Han L, Wang Q, Jiang K, Kang C, and Zhang Q. 2016. "AURKA Induces EMT by Regulating Histone Modification through Wnt/β-Catenin and PI3K/AKT Signaling Pathway in Gastric Cancer." *Oncotarget* 7:33152–64. doi: 10.18632/oncotarget.8888.
- Lopez-Rubio JJ, Gontijo AM, Nunes MC, Issar N, Rivas RH, and Scherf A. 2007. "5' Flanking Region of Var Genes Nucleate Histone Modification Patterns Linked to Phenotypic Inheritance of Virulence Traits in Malaria Parasite." *Mol. Microbiol* 66:1296–1305. doi: 10.1111/j.1365-2958.2007.06009.x.
- Lopez-Rubio JJ, Mancio-Silva L, and Scherf A. 2009. "Genome-Wide Analysis of Heterochromatin Associates Clonally Variant Gene Regulation with Perinuclear Repressive Centers in Malaria Parasites." *Cell Host Microbe* 5:179–90. doi: 10.1016/j.chom.2008.12.012.
- Luah YH, Chaal BK, Ziying Ong E, and Bozdech Z. 2010. "A Moonlighting Function of *Plasmodium falciparum* Histone 3, Mono-Methylated at Lysine 9?" *PLoS One* 5:e10252. doi: 10.1371/journal.pone.0010252.
- Martens S, Parvanova I, Zerrahn J, Griffiths G, Schell G, Reichmann G, and Howard JC. 2005. "Disruption of *Toxoplasma gondii* Parasitophorous Vacuoles by the Mouse P47-Resistance GTPases." *PLoS Pathog* 1:e24. doi: 10.1371/journal.ppat.0010024.
- Matos LVS, McEvoy J, Tzipori S, Brescian KDS, and Widmer G. 2019. "The Transcriptome of *Cryptosporidium* Oocysts and Intracellular Stages." *Sci Rep* 9:7859. doi: https://doi.org/10.1038/s41598-019-44289-x.
- McCole DF, Eckmann L, Laurent F, and Kagnoff MF. 2000. "Intestinal Epithelial Cell Apoptosis Following *Cryptosporidium parvum* Infection." *Infect Immun* 68:1710–13. doi: 10.1128/IAI.68.3.1710-1713.2000.
- Mele, Raffaella, Maria Angeles Gomez Morales, Fabio Tosini, and Edoardo Pozio. 2004. "Cryptosporidium parvumat Different Developmental Stages Modulates Host Cell Apoptosis in Vitro." *Infection and Immunity* 72(10):6061–67. doi: 10.1128/IAI.72.10.6061-6067.2004.
- Miao, J, Fan Q, Cui L, and Li J. 2006. "The Malaria Parasite *Plasmodium falciparum* Histones: Organization, Expression, and Acetylation." *Gene* 369:53–65.
- Mostafa, Nahed E., Enas F. Abdel Hamed, Eman M. Fawzy, Rabab S. Zalat, Hayam E. Rashed, and Salem Y. Mohamed. 2018. "The New Trend in the Treatment of

References

Experimental Cryptosporidiosis and the Resulting Intestinal Dysplasia." *Colorectal Cancer* 7(4):CRC06. doi: 10.2217/crc-2018-0008.

- Murakoshi F, Bando H, Sugi T, Adeyemi OS, Nonaka M, Nakaya T, and Kato K. 2020. "Nullscript Inhibits *Cryptosporidium* and *Toxoplasma* Growth." *Int J Parasitol Drugs Drug Resist* . 14:159–66. doi: 10.1016/j.ijpddr.2020.10.004.
- Nagaraj S. and Gabrilovich DI. 2008. "Tumor Escape Mechanism Governed by Myeloid-Derived Suppressor Cells." *Cancer Res* 68:2561–63. doi: 10.1158/0008-5472.can-07-6229.
- Nakagun, Shotaro, Noriyuki Horiuchi, Miki Sugimoto, Sohei Tomikawa, Kenichi Watanabe, and Yoshiyasu Kobayashi. 2017. "Proventriculitis Associated with *Cryptosporidium Baileyi* in a Snowy Owl (*Bubo Scandiacus*) and Its Epidemiological Investigation." *Journal of Parasitology* 103(5):451–57. doi: 10.1645/17-54.
- Nishioka K, Rice JC, Sarma K, Erdjument-Bromage H, Werner J, Wang Y, Chuikov S, Valenzuela P, Tempst P, and Steward R. 2002. "PR-Set7 Is a Nucleosome-Specific Methyltransferase That Modifies Lysine 20 of Histone H4 and Is Associated with Silent Chromatin." *Mol Cell* 9:1201–13. doi: 10.1016/s1097-2765(02)00548-8.
- Oberstaller J, Pumpalova Y, Schieler A, Llinás M, and Kissinger JC. 2014. "The *Cryptosporidium parvum* ApiAP2 Gene Family: Insights into the Evolution of Apicomplexan AP2 Regulatory Systems." *Nucleic Acids Res* 42:8271–84. doi: 10.1093/nar/gku500.
- Olliaro P. 2008. "Mortality Associated with Severe *Plasmodium falciparum* Malaria Increases with Age." *Clin Infect Dis* 47:158–60. doi: 10.1086/589288.
- Peddareddigari VG, Wang D, and DuBois RN. 2010. "The Tumor Microenvironment in Colorectal Carcinogenesis." *Cancer Microenviron* 3:149–66. doi: 10.1007/s12307-010-0038-3.
- Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH, and Ferrin TE. 2021. "UCSF ChimeraX: Structure Visualization for Researchers, Educators, and Developers." *Protein Sci* 30:70–82. doi: 10.1002/pro.3943.
- Pflügler S, Svinka J, Scharf I, Crncec I, Filipits M, Charoentong P, Tschurtschenthaler M, Kenner L, Awad M, and Stift J. 2020. "IDO1+ Paneth Cells Promote Immune Escape of Colorectal Cancer." *Commun. Biol* 3:252. doi: 10.1038/s42003-020-0989-y.
- Pivovarova Y, Liu J, Lesigang J, Koldyka O, Rauschmeier R, Hu K, and Dong G. 2018. "Structure of a Novel Dimeric SET Domain Methyltransferase That Regulates Cell Motility." *J Mol Biol* 430:4209–29. doi: 10.1016/j.jmb.2018.08.017.
- Pollock WB, Rosell FI, Twitchett MB, Dumont ME, and Mauk AG. 1998. "Bacterial Expression of a Mitochondrial Cytochrome c. Trimethylation of Lys72 in Yeast Iso-1-Cytochrome c and the Alkaline Conformational Transition." *Biochemistry* 37:6124–31. doi: 10.1021/bi972188d.

References

- Porras-Yakushi TR, Whitelegge JP, and Clarke S. 2007. “Yeast Ribosomal/Cytochrome c SET Domain Methyltransferase Subfamily: Identification of Rpl23ab Methylation Sites and Recognition Motifs.” *J Biol Chem* 282:12368–76. doi: 10.1074/jbc.M611896200.

- Ramasundara M, Leach ST, Lemberg DA, and Day SA. 2009. “Defensins and Inflammation: The Role of Defensins in Inflammatory Bowel Disease.” *J Gastroenterol Hepatol* . 24:202–8. doi: 10.1111/j.1440-1746.2008.05772.x.

- Ramirez NE, Ward LA, and Sreevatsan S. 2004. “A Review of the Biology and Epidemiology of Cryptosporidiosis in Humans and An-Imals.” *Microbes Infect.* 6:773–85.

- Razakandrainibe R, Kubina S, Costa D, Robinson G, Carbona SL, Aubert D, David A, Gargala G, Villena I, Favennec L, and Chalmers RM. 2020. “Evaluation of a Modified Method for the Detection of *Cryptosporidium* Oocysts on Spinach Leaves.” *Food Waterborne Parasitol* 21:e00097. doi: 10.1016/j.fawpar.2020.e00097.

- Rea S, Eisenhaber F, O’Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, and Jenuwein T. 2000a. “Regulation of Chromatin Structure by Site-Specific Histone H3 Methyltransferases.” *Nature* 406:593–99. doi: 10.1038/35020506.

- Rea S, Eisenhaber F, O’Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, and Jenuwein T. 2000b. “Regulation of Chromatin Structure by Site-Specific Histone H3 Methyltransferases.” *Nature* 406:593–99. doi: 10.1038/35020506.

- Ricciardi M, Zanotto M, Malpeli G, Bassi G, Perbellini O, Chilosi M, Bifari F, and Krampera M. 2015. “Epithelial-to-Mesenchymal Transition (EMT) Induced by Inflammatory Priming Elicits Mesenchymal Stromal Cell-like Immune-Modulatory Properties in Cancer Cells.” *Br. J. Cancer* 112:1067–75. doi: 10.1038/bjc.2015.29.

- Rider SD Jr and Zhu G. 2009. “An Apicomplexan Ankyrin-Repeat Histone Deacetylase with Relatives in Photosynthetic Eukaryotes.” *Int J Parasitol* 39:747–54. doi: 10.1016/j.ijpara.2008.11.012.

- Rider SD Jr and Zhu G. 2010. “*Cryptosporidium*: Genomic and Biochemical Features.” *Exp Parasitol* 124:2–9. doi: 10.1038/ncb1151.

- Rodrigues-Pousada CA, Nevitt T, Menezes R, Azevedo D, Pereira J, and Amaral C. 2004. “Yeast Activator Proteins and Stress Response: An Overview.” *FEBS Lett* . 567:80–85. doi: 10.1016/j.febslet.2004.03.119.

- Ryan U, Hijjawi N, and Xiao L. 2018. “Foodborne Cryptosporidiosis.” *Int. J. Parasitol* 48:1–12.

- Ryan U, Paparini A, Monis P, and Hijjawi N. 2016. “*Cryptosporidium* Is a Gregarine: What Are the Implications for the Water Industry?” *Water Res* 105:305–13. doi: 10.1016/j.watres.2016.09.013.

References

- Ryan U, Zahedi A, Feng Y, and Xiao L. 2021. “An Update on Zoonotic *Cryptosporidium* Species and Genotypes in Humans.” *Animals (Basel)* 11:3307. doi: 10.3390/ani11113307.
- Ryan, Una, Ronald Fayer, and Lihua Xiao. 2014. “*Cryptosporidium* Species in Humans and Animals: Current Understanding and Research Needs.” *Parasitology* 141(13):1667–85. doi: 10.1017/S0031182014001085.
- Sablet T, Potiron L, Marquis M, Bussière FI, Lacroix-Lamandé S, and Laurent F. 2016. “*Cryptosporidium* parvumIncreases the Intestinal Permeability through Interaction with Epithelial Cells and IL-1β and TNF-α Released by Inflammatory Monocytes.” *Cell Microbiol* 18:1871–80. doi: 10.1111/cmi.12632.
- Saksouk N, Bhatti MM, Kieffer S, Smith AT, Musset K, Garin J, Sullivan WJ Jr, Cesbron-Delauw MF, and Hakimi MA. 2005. “Histone-Modifying Complexes Regulate Gene Expression Pertinent to the Differentiation of the Protozoan Parasite *Toxoplasma gondii*.” *Mol Cell Biol* 25:10301–14. doi: 10.1128/MCB.25.23.10301-10314.2005.
- Santos-Rosa H, Schneider R, Bannister AJ, Sherriff J, Bernstein BE, Tolga Emre NC, Schreiber SL, Mellor J, and Kouzarides T. 2002. “Active Genes Are Tri-Methylated at K4 of Histone H3.” *Nature* 419:407–11. doi: 10.1038/nature01080.
- Sateriale A, Gullicksrud JA, McLeod BI, Kugler EM, Henao-Mejia J, Zhou T, Ring AM, Brodsky IE, Hunter CA, and Streipen B. 2021. “The Intestinal Parasite *Cryptosporidium* Is Controlled by an Enterocyte Intrinsic Inflammasome That Depends on NLRP6.” *Proc Natl Acad Sci U S A* 118:e2007807118. doi: 10.1073/pnas.2007807118.
- Sateriale A, Šlapeta J, Baptista R, Engiles JB, Gullicksrud JA, Herbert GT, Brooks CF, Kugler EM, Kissinger JC, and Hunter CA. 2019. “A Genetically Tractable, Natural Mouse Model of Cryptosporidiosis Offers Insights into Host Protective Immunity.” *Cell Host Microbe* 26:135–46. doi: 10.1016/j.chom.2019.05.006.
- Sautel CF, Cannella D, Bastien O, Kieffer S, Aldebert D, Garin J, Tardieux I, Belrhali H, and Hakimi MA. 2007a. “SET8-Mediated Methylations of Histone H4 Lysine 20 Mark Silent Heterochromatic Domains in Apicomplexan Genomes.” *Mol Cell Biol* 27:5711–24. doi: 10.1128/MCB.00482-07.
- Sautel CF, Cannella D, Bastien O, Kieffer S, Aldebert D, Garin J, Tardieux I, Belrhali H, and Hakimi MA. 2007b. “SET8-Mediated Methylations of Histone H4 Lysine 20 Mark Silent Heterochromatic Domains in Apicomplexan Genomes.” *Mol Cell Biol* 27(16):5711–24. doi: 10.1128/MCB.00482-07.
- Sautel CF, Ortet P, Saksouk N, Kieffer S, Garin J, Bastien O, and Hakimi MA. 2009a. “The Histone Methylase KMTox Interacts with the Redox-Sensor Peroxiredoxin-1 and Targets Genes Involved in *Toxoplasma gondii* Antioxidant Defences.” *Mol. Microbiol* 71:212–26. doi: 10.1111/j.1365-2958.2008.06519.x.

References

- Sautel CF, Ortet P, Saksouk N, Kieffer S, Garin J, Bastien O, and Hakimi MA. 2009b. "The Histone Methylase KMTox Interacts with the Redox-Sensor Peroxiredoxin-1 and Targets Genes Involved in *Toxoplasma gondii* Antioxidant Defences." *Mol Microbiol* 77:212–26. doi: 10.1111/j.1365-2958.2008.06519.x.
- Sawant M, Baydoun M, Creusy C, Chabé M, Viscogliosi E, Certad G, and Benamrouz-Vanneste S. 2020. "*Cryptosporidium* and Colon Cancer: Cause or Consequence?" *Microorganisms* 8:1665. doi: 10.3390/microorganisms8111665.
- Sawant M, Benamrouz-Vanneste S, Mouray A, Bouquet P, Gantois N, Creusy C, Duval E, Mihalache A, Gosset P, Chabé M, Hot D, Viscogliosi E, and Certad G. 2021. "Persistent *Cryptosporidium parvum* Infection Leads to the Development of the Tumor Microenvironment in an Experimental Mouse Model: Results of a Microarray Approach." *Microorganisms* 9:2569. doi: 10.3390/microorganisms9122569.
- Schneider R, Bannister AJ, and Kouzarides T. 2002. "Unsafe SETs: Histone Lysine Methyltransferases and Cancer." *Trends Biochem. Sci* 27:396–402. doi: 10.1016/s0968-0004(02)02141-2.
- Sever, Richard, and Joan S. Brugge. 2015. "Signal Transduction in Cancer." *Cold Spring Harbor Perspectives in Medicine* 5(4). doi: 10.1101/CSHPERSPECT.A006098.
- Shurin MR. 2018. "Osteopontin Controls Immunosuppression in the Tumor Microenvironment." *J. Clin. Investig* 128:5209–12. doi: 10.1172/jci124918.
- Sims RJ III, Nishioka K, and Reinberg D. 2003. "Histone Lysine Methylation: A Signature for Chromatin Function." *Trends Genet.* 19:629–39. doi: 10.1016/j.tig.2003.09.007.
- Sivagurunathan S, Heaslip A, Liu J, and Hu K. 2013. "Identificaiton of Functional Modules of AKMT, a Novel Lysine Methyltransferase Regulating the Motility of *Toxoplasma gondii*." *Mol Biochem Parasitol* 189:43–53. doi: 10.1016/j.molbiopara.2013.05.004.
- Solinas G, Germano G, Mantovani A, and Allavena P. 2009. "Tumor-Associated Macrophages (TAM) as Major Players of the Cancer-Related Inflammation." *J. Leukoc. Biol* 86:1065–73. doi: 10.1189/jlb.0609385.
- Southhall SM, Wong PS, Odho Z, Roe SM, and Wilson JR. 2009. "Structural Basis for the Requirement of Additional Factors for MLL1 SET Domain Activity and Recognition of Epigenetic Marks." *Mol Cell* 33:181–91. doi: 10.1016/j.molcel.2008.12.029.
- de Souza, Lenice do Rosário, Maria Aparecida M. Rodrigues, José Morceli, Raphael Kemp, and Rinaldo Poncio Mendes. 2004. "Cryptosporidiosis of the Biliary Tract Mimicking Pancreatic Cancer in an AIDS Patient." *Revista Da Sociedade Brasileira de Medicina Tropical* 37(2):182–85.
- Stedman E. 1950. "Cell Specificity of Histones." *Nature* 166:780–81. doi: 10.1038/166780a0.

References

- Stephens, J., M. Cosyns, M. Jones, and A. R. Hayward. 1999. “Liver and Bile Duct Pathology Following *Cryptosporidium parvum* Infection of Immunodeficient Mice.” *Hepatology (Baltimore, Md.)* 30(1):27–35. doi: 10.1002/hep.510300138.
- Strahl BD and Allis CD. 2000. “The Language of Covalent Histone Modifications.” *Nature* 403:41–45. doi: 10.1038/47412.
- Sullivan WJ., Jr and Hakimi MA. 2006. “Histone Mediated Gene Activation in *Toxoplasma gondii*.” *Mol Biochem Parasitol* 148:109–16. doi: 10.1016/j.molbiopara.2006.03.010.
- Sullivan WJ Jr, Naguleswaran A, and Angel SO. 2006. “Histones and Histone Modifications in Protozoan Parasites.” *Cell Microbiol* 8:1850–61. doi: 10.1111/j.1462-5822.2006.00818.x.
- Tandel J, English ED, Sateriale A, Gullicksrud JA, Beiting DP, Sullivan MC, Pinkston B, and Striepen B. 2019. “Life Cycle Progression and Sexual Development of the Apicomplexan Parasite *Cryptosporidium parvum*.” *Nat Microbiol* 4:2226–36. doi: 10.1038/s41564-019-0539-x.
- Templeton TJ, Iyer LM, Anantharaman V, Enomoto S, Abrahante JE, Subramanian GM, Hoffman SL, Abrahamsen MS, and Aravind L. 2004a. : “: Comparative Analysis of Apicomplexa and Genomic Diversity in Eukaryotes.” *Genome Res.* 14(1686–1695). doi: 10.1101/gr.2615304.
- Templeton TJ, Iyer LM, Anantharaman V, Enomoto S, Abrahante JE, Subramanian GM, Hoffman SL, Abrahamsen MS, and Aravind L. 2004b. “Comparative Analysis of Apicomplexa and Genomic Diversity in Eukaryotes.” *Genome Res.* 14(9):1686–95. doi: doi: 10.1101/gr.2615304.
- Tenter AM, Heckeroth AR, and Weiss LM. 2000. “*Toxoplasma gondii:* From Animals to Humans.” *Int J Parasitol* . 30:1217–58. doi: 10.1016/s0020-7519(00)00124-7.
- Terrell, Scott P., Elizabeth W. Uhl, and Richard S. Funk. 2003. “Proliferative Enteritis in Leopard Geckos (Eublepharis Macularius) Associated with *Cryptosporidium* Sp. Infection.” *Journal of Zoo and Wildlife Medicine : Official Publication of the American Association of Zoo Veterinarians* 34(1):69–75. doi: 10.1638/1042-7260(2003)34[0069:PEILGE]2.0.CO;2.
- Tolias, K. F., L. C. Cantley, and C. L. Carpenter. 1995. “Rho Family GTPases Bind to Phosphoinositide Kinases.” *The Journal of Biological Chemistry* 270(30):17656–59. doi: 10.1074/JBC.270.30.17656.
- Tretina K, Gotia HT, Mann DJ, and Silva JC. 2015. “*Theileria*-Transformed Bovine Leukocytes Have Cancer Hallmarks.” *Trends Parasito* 31:306–14.
- Trojer P and Reinberg D. 2006. “Histone Lysine Demethylases and Their Impact on Epigenetics.” *Cell* 125:213–17. doi: 10.1016/j.cell.2006.04.003.

References

- Vanagas L, Jeffers V, Bogado SS, Dalmasso MC, Sullivan Jr WJ, and Angel SO. 2012. "*Toxoplasma* Histone Acetylation Remodelers as Novel Drug Targets." *Expert Rev Anti Infect Ther* 10(10):1189–1201. doi: 10.1586/eri.12.100.

- Villares M, Berthelet J, and Weitzman JB. 2020. "The Clever Strategies Used by Intracellular Parasites to Hijack Host Gene Expression." *Semin Immunopathol* 42:215–26. doi: 10.1007/s00281-020-00779-z.

- Vinayak S, Pawlowic MC, Sateriale A, Brooks CF, Studstill CJ, Bar-Peled Y, Cipriano MJ, and Striepen B. 2015. "Genetic Modification of the Diarrhoeal 866 Pathogen *Cryptosporidium parvum*." *Nature* 523:477–80. doi: 10.1038/nature14651.

- Vivanco, Igor, and Charles L. Sawyers. 2002. "The Phosphatidylinositol 3-Kinase AKT Pathway in Human Cancer." *Nature Reviews. Cancer* 2(7):489–501. doi: 10.1038/NRC839.

- Wagner EJ and Carpenter PB. 2012. "Understanding the Language of Lys36 Methylation of Histone H3." *Nat Rev Mol Cell Bio* 13:115–26. doi: 10.1038/nrm3274.

- Wang D and Dubois RN. 2015. "Immunosuppression Associated with Chronic Inflammation in the Tumor Microenvironment." *Carcinogenesis* 36:1085–93. doi: 10.1093/carcin/bgv123.

- Wang Y, Gong A-Y, Ma S, Chen X, Strauss-Soukup JK, and Chen X-M. 2017. "Delivery of Parasite Cdg7_Flc_0990 RNA Transcript into Intestinal Epithelial Cells during *Cryptosporidium parvum* Infection Suppresses Host Cell Gene Transcription through Epigenetic Mechanisms." *Cell Microbiol* 19:e12760.

- Whiteman, David C., and Louise F. Wilson. 2016. "The Fractions of Cancer Attributable to Modifiable Factors: A Global Review." *Cancer Epidemiology* 44:203–21.

- Williams CJ, Head JJ, Moriarty NW, Prisant MG, Videau LW, Deis LN, Verma V, Keddy DA, Hintze BJ, and Chen VB. 2018. "MolProbity: More and Better Reference Data for Improved All-atom Structure Validation." *Protein Sci* 27:293-315. doi: 10.1002/pro.3330.

- Winston F and Allis CD. 1999. "The Bromodomain: A Chromatin-Targeting Module?" *Nat Struct Biol* 6:601–4. doi: 10.1038/10640.

- Xiao B, Jing C, Kelly G, Walker PA, Muskett FW, Frenkiel TA, Martin SR, Sarma K, Reinberg D, Gamblin SJ, and Wilson JR. 2005. "Specificity and Mechanism of the Histone Methyltransferase Pr-Set7." *Genes Dev* 19:1444–54. doi: 10.1101/gad.1315905.

- Xu P, Widmer G, Wang Y, Ozaki LS, Alves JM, Serrano MG, Puiu D, Manque P, Akiyoshi D, and Mackey AJ. 2004. "The Genome of Cryptosporidium Hominis." *Nature* 431(7012):1107–12. doi: 10.1038/nature02977.

References

- Yang X, Guo Y, Xiao L, and Feng Y. 2021. "Molecular Epidemiology of Human Cryptosporidiosis in Low- and Middle-Income Countries." *Clin. Microbiol. Rev.* 34:e00087-19. doi: 10.1128/CMR.00087-19.
- Zhang, Nan, Xiuyan Yu, Hongbo Zhang, Lianying Cui, Xiaoou Li, Xiaowei Zhang, Pengtao Gong, Jianhua Li, Ziyi Li, Xiaocen Wang, Xin Li, Ting Li, Xiaofeng Liu, Yanhui Yu, and Xichen Zhang. 2020. "Prevalence and Genotyping of *Cryptosporidium parvum* in Gastrointestinal Cancer Patients." *Journal of Cancer* 11(11):3334–39. doi: 10.7150/jca.42393.
- Zhang T, Cooper S, and Brockforff N. 2015. "The Interplay of Histone Modifications - Writers That Read." *EMBO Rep* 16:1467–81. doi: 10.15252/embr.201540945.
- Zhang X, Tamaru H, Khan SI, Horton JR, Keefe LJ, Selker EU, and Cheng X. 2002. "Structure of the Neurospora SET Domain Protein DIM-5, a Histone H3 Lysine Methyltransferase." *Cell* 111:117–27. doi: 10.1016/s0092-8674(02)00999-6.
- Zhang X, Yang Z, Khan SI, Horton JR, Tamaru H, Selker EU, and Cheng X. 2003. "Structural Basis for the Product Specificity of Histone Lysine Methyltransferases." *Mol Cell* 12:177–85.
- Zhang Y. 2006. "It Takes a PHD to Interpret Histone Methylation." *Nat Struct Mol Biol* . 13:572–74. doi: 10.1038/nsmb0706-572.
- Zhu W, Li J, Pappoe F, Shen J, and Yu Li. 2019. "Strategies Developed by *Toxoplasma gondii* to Survive in the Host." *Front. Microbiol* 10:899. doi: 10.3389/fmicb.2019.00899.