Project n°: 221453

Project Acronym: APICOLIPID

Project Full Name:

Lipidomic Analysis and functional study of the lipid biosynthesis of the plastid of Apicomplexa parasites

Marie Curie Actions

OIF Final Activity and Management Report

Period covered: from October 2008 to October 2011

Period number: 1 and 2

Start date of project: 01/10/2008

Project coordinator name: Eric Maréchal

Project coordinator organisation name: CNRS

Date of preparation: 25/11/11

Date of submission (SESAM):

Duration: 3 years

Version:

1. FINAL PUBLISHABLE SUMMARY REPORT

Background and objectives of the Apicolipid project

Apicomplexa is a phylum of obligate intracellular parasites, including pathogens of medical and veterinary importance such as toxoplasmosis and malaria. Toxoplasmosis affects up to one third of the world's human population and can have dramatic consequences in immunodeprived patients and pregnant women. Malaria is one the deadliest human disease, infecting about 225 million people each year and resulting in up to 1 million deaths, predominantly children in sub saharan Africa and southeast Asia (WHO Report Dec. 2010). There is no current vaccine against malaria and current treatments are losing effectiveness due to increasing parasite drug resistance, even to front line drugs.Therefore, there is a pressing need for the development of new efficient molecules against the diseases, especially malaria.
In contrast to pathogenic bacteria, Apicomplexa parasites are unicellular eukaryotes and share a wide range of metabolic pathways with their animal hosts, making therapeutic target development difficult. However, Apicomplexa harbour a relict non-photosynthetic plastid, the apicoplast, which has been acquired by the secondary endosymbiosis of a red alga (McFadden et al. 1996). The apicoplast is involved in unique and vital biological processes for the parasite (Fichera and Roos 1997). Due to its plant origin, the apicoplast thus represents a potential drug target against Apicomplexa related diseases. Beside the loss of photosynthetic capacities, the apicoplast is otherwise metabolically similar to plant and algal chloroplast and thus unique to the parasite. The apicoplast thus represents a novel potential target against Apicomplexa (Botté et al. 2011).
As a consequence of its algal origin, the apicoplast hosts a complete prokaryotic type II fatty acid synthesis pathway or FASII pathway (Waller et al. 1998). Fatty acids are the essential building blocks for the synthesis of membrane lipids. Very little is known on the exact nature and fate of the fatty acids generated by the apicoplast FASII pathway. However, previous studies indicate that the FASII pathway is essential for the parasite and may supply fatty acids for the biogenesis of the apicoplast as well as extra-plastidial membranes (Mazumdar et al. 2006, Vaughan et al. 2008, Yu et al. 2009). Beyond being able to synthesize fatty acids via its FASII, the apicoplast might be able to utilize them. Indeed, a set of acyltransferases, catalysing the synthesis of phosphatidic acid (PA), the central precursor for most membrane lipids, are predicted to be localised to the apicoplast (Ralph et al. 2004). Therefore, the apicoplast may play a central part in the lipid synthesis and homeostasis, which are crucial for the parasite division, proliferation and survival.
Our project aims to understand the apicoplast lipid metabolism and how the organelle is involved in the parasite's vital demand for membrane syntheses. Our comprehensive study is divided in three objectives. The first objective intends to purify the apicoplast and establish the first lipidome of the organelle via a novel isolation protocol combined to metabolomic approaches. The second objective is to identify the apicoplast lipid products and assess their sub-cellular fate via metabolic labelling, using the lipid profiles obtained in the first objectives as baselines. The third objective is to characterize the enzymes responsible for the synthesis of PA, i.e. two acyltransferases homolog to plant and algal chloroplast ATS1 and ATS2. We will try to understand if the apicoplast is the site for the initial step of membrane lipid synthesis and determine if these enzymes are potential drug targets. Our investigation goes beyond understanding the apicoplast metabolism and seeks to establish the raison d'être of the organelle.

Results

Apicoplast purification and lipidomic profile

We made a major technical breakthrough in our quest to understand how parasite apicoplast works. No suitable procedure existed to isolate the plastids of apicomplexan parasites. We have developed a unique strategy to isolate the organelle in the malaria parasite *P. falciparum*. The protocol is based on the immunopurification of the apicoplast expressing an epitope-tag fused to the C-terminus of one of its outer

membrane proteins. Blood stage malaria parasites are first separated from their host cells, then broken up and finally mixed with magnetic beads coated with antibodies directed against the epitope-tag. Beads with bound apicoplasts are retrieved with a magnet and the sample purity is vetted by immunofluorescence, electron microscopy and western blotting. Previous attempts on apicoplast purification mainly failed due to their interaction and the density properties that mitochondrion and apicoplast share, thus making them virtually impossible to separate. Here, we show for the first time a clear enrichment of the apicoplast, associated with depletion in mitochondria presence using our purification method.

Subsequently, we scaled up the purification procedure in order to obtain enough biological material for lipidomic analysis. In collaboration with Metabolomics Australia and Prof McConville (Bio21, University of Melbourne), we analyzed the lipid content of isolated apicoplasts and whole parasites free of their host cell. We used GC-MS analysis for fatty acid profiling in conjunction with the novel LC-MS/MS analysis for whole lipidomic profiling. When compared to whole parasites, apicoplast are enriched in saturated fatty acids, especially stearic acid (C18:0). We also identified 194 molecular species of lipids belonging to nine major lipid classes present in the apicoplast membranes. We quantified these lipid classes and obtained the first lipid profile of the apicoplast, surprisingly lacking galactolipids, which is the predominant lipid class in chloroplast membranes (Botté et al. 2005). Very interestingly, apicoplast membranes are containing cholesterol and cholesterol esters, which do not occur in plant chloroplasts as plants lack a pathway to synthesise cholesterol. Considering that malaria parasites also lack cholesterol synthesis, we assume this cholesterol is scavenged from the host erythrocyte for incorporation into apicoplast membranes. This is obviously a major departure from how things work in plant plastids and a potentially good target for drug intervention. These results demonstrate that the apicoplast has a very unique lipidome for a plastid and that it contains a complex set of lipids, some diverted from the host, some remodelled by the parasites, and perhaps some from its *de novo* synthesis machinery (Botté et al. submitted). Our purification apicoplast protocol was successfully applied in *Toxoplasma gondii*, the causative agent of toxoplasmosis. Indeed, our results indicate that, for the first time, we isolated its apicoplast separated from the mitochondrion as confirmed by western blot analysis and immunofluorescence assays. We are currently conducting a lipidomic analysis of *T. gondii*'s apicoplast. *T. gondii* lipid metabolism differs from the malaria parasite since the apicoplast FASII is always essential during the acute phase of the disease within the human/mammal host. Therefore, it is important to determine how the apicoplast in involved in the *T. gondii* lipid synthesis as it may reveal major differences between the two parasites and the way to target and inhibit the pathway.

Combining both results obtained from previous reports (Maréchal et al. 2002, Bisanz et al. 2006, Welti et al. 2007, Botté et al. 2008) and our own lipid analysis of purified apicoplasts from *P. falciparum* (objective 1 of this proposal), the question on galactolipid synthesis in Apicomplexa therefore remains unsolved. Are galactolipids still generated via a plant-like pathway using highly divergent enzymes or has the pathway been modified when the apicoplast lost its photosynthetic capacities? Are the galactolipids detected in the Apicomplexa unrelated to the original endosymbiotic red alga? Very recently, *Chromera velia*, a photosynthetic protist closely related to Apicomplexa was discovered as a coral symbiont in the Sydney Bay (Moore et al. 2008). *Chromera velia* harbours an apicoplast that is still photosynthetic and therefore constitutes a very interesting model to study secondary plastids' evolution from autotrophic to heterotrophic state as found in Apicomplexa. We report the unambiguous characterization of plant-like galactolipids in *C. velia* via HPTLC, GC-MS and LC/MS-MS approaches and determined their localization by immunocytochemistry. We characterized *C. velia* galactolipid synthesis pathway as a plant-like pathway by metabolic labelling. Finally, in collaboration with Dr Keeling's laboratory (University of British Columbia, Canada), which is currently developing an EST database on *C. Velia*, we identified the candidate genes encoding for the galactosyltransferases responsible for galactolipid synthesis in *C. velia* (Botté et al. 2011).

Apicoplast lipid synthesis

In order to characterize the lipids that are synthesized by the apicoplast, we performed metabolic labelling with specific substrates followed by GC-MS analysis in *P. falciparum* blood stages. Acetyl-CoA is the major substrate for fatty acids synthesis via the FASII pathway, and can only be generated upon the import of phosphoenol pyruvate (PEP), an intermediate of the parasite glycolysis, into the apicoplast. Therefore, we decided to use [^{13}C] labelled glucose, which is indispensible for the parasite and actively pumped into the parasite's cytosol. This approach allowed to specifically following the incorporation of labelled glucose into neo-synthesized fatty acids, which could then be discriminated via GC-MS. Recent studies showed that apicoplast FASII pathway was rather crucial during liver stages than intra-erythrocytic life stages (Yu et al. 2008, Vaughan et al. 2009), however, they were not able to determine what fatty acids was produced via the pathway. Our analysis showed that the apicoplast FASII was active during erythrocytic stages and predominantly synthesized short fatty acid chains at low levels. We are currently applying our analysis method to other life stages as well as purified apicoplast fraction from both *P. falciparum* and *T. gondii*, to understand the apicoplast global input in terms of fatty acid synthesis for the parasite membrane synthesis.

The apicoplast as an important site for phospholipid precursor synthesis

Replication of the parasite in the Human host requires the obtention of sufficient lipids for membrane biogenesis. The majority of membrane lipids (glycerolipids) are synthesized from a unique precursor: phosphatidic acid. Analyses of *Plasmodium spp*. genomes indicate the presence of two putative acyltransferases, homolog to plants ATS1 and ATS2, in the apicoplast of the parasite. We investigated the first enzyme, ATS1, of the proposed apicoplast phosphatidic acid synthesis pathway in both *Plasmodium falciparum* and *Toxoplasma gondii*. We confirmed the localisation of ATS1 in the apicoplast of both parasites using GFP fusion and epitope-tagged versions of the protein. The acyltransferase activity of the enzyme was then confirmed by complementation assays performed in *E. coli*. Importantly, we engineered a conditional null-mutant in *T. gondii* and showed that ATS1 is essential for parasite growth by disruption of the apicoplast biogenesis. Together these findings provide the first evidences to support the presence of a novel phosphatidic acid synthesis pathway in the apicoplast that can be investigated as a potential drug target. We are currently investigating the role of PfATS1 in both blood and liver stages. We have also engaged a similar approach to study the localisation and function of ATS2.

USE AND DISSEMINATION OF FOREGROUND

Section A (public) – DISSEMINATION MEASURES

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- **Dissemination activities**

Scientific publications

- Dubots E, **Botté CY**, Boudière L, Yamaryo-Botté Y, Jouhet J, Maréchal E, Block MA. (2011). *Role of Phosphatidic acid in plant galactolipidsynthesis.* ***Biochimie.*** In press (IF = 3.787)
- **Botté CY**, Yamaryo-Botté Y, Janouškovec J, Rupasinghe T, Keeling P, Crellin P, Coppel R, Maréchal E, McConville M, McFadden GI. *Identification of plant-like galactolipids in the apicoplast of Chromera velia, a photosynthetic Apicomplexa*. ***J Biol Chem.*** 2011 Aug 26;286(34):29893-903. (IF = 5.328)
- **Botté CY**, Deligny M, Roccia A, Bonneau A L, Saïdani N, Hardré-Liénard H, Aci S, Jouhet J, Dubots E, Loizeau K, Bastien O, Bréhélin L, Joyard J, Falconet D, Block M, Rousseau, B., Lopez R, Maréchal E. *Chemical inhibitors of monogalactosyldiacylglycerol synthases in Arabidopsis thaliana.* ***Nat Chem Biol.*** 2011 Sep 25;7(11):834-842. doi: 10.1038/nchembio.658 (IF =16.058)
- **Botté CY**, Dubar F, McFadden GI, Maréchal E, Biot C. *Plasmodium falciparum apicoplast drugs: targets or off-targets?* ***Chemical Reviews.*** 2011 Oct 25. [Epub ahead of print] (IF = 33.033)
- MacRae JI, Maréchal E, Biot C, **Botté CY**. *The Apicoplast: a key plant feature and potential target to cure malaria?* **Curr Pharm Design**. [Accepted with minor modification] (IF =4.774)
- **Botté CY**, Mullin KA, Yamaryo-BottéY, Rupasinghe T, Spurck T., Kalanon M, Crellin P, Coppel R, Maréchal E, McConville M and McFadden GI. *Purification and lipidomic analysis of the malaria parasite apicoplast unravel a unique lipid composition*. Submitted to ***J Cell Biol*** (if accepted IF = 9.575)
- Biot C, Castro W, **Botté CY**, Navarro M. *The Therapeutic potential of metal-based antimalarial. Implications for the mechanisms of action.* Submitted to ***Dalton T*** (if accepted IF =3.647)

Oral presentations in international conferences

-Malaria in Melbourne. October 26th and 26th 2009, Melbourne, Australia

Botté CY, Mullin KA,Yamaryo-Botté Y, Rupasinghe T, Shears MJ, Spurck T, Coppel RL, McConville MJ, Eric Maréchal, McFadden GI. "*Lipidomic analysis and functional study of the lipid biosynthesis of the malaria apicoplast"*

- International society of evolutionary protistology, ISEP XVIII, 2-7 July 2010, Kanazawa, Japan

Botté CY, Yamaryo-Botté Y, Janouskovec J., Keeling P.Coppel RL, McConville MJ, Maréchal E, McFadden GI. *"Identification of plant-like galactolipids in the apicoplast of Chromera velia, an ancestor of malaria parasites"*

- International symposium of plant lipids, ISPL 2010 11-16 july, Cairns, Australia

Botté CY, Mullin KA,Yamaryo-Botté Y, Rupasinghe T, Shears MJ, Spurck T, Coppel RL, McConville MJ, Eric Maréchal, McFadden GI. "*Lipidomic analysis and functional study of the lipid biosynthesis of the malaria apicoplast"*

- International congress of parasitology, ICOPA XII, 15-20 August 2010 Melbourne, Australia

Botté CY, Mullin KA,Yamaryo-Botté Y, Rupasinghe T, Shears MJ, Spurck T, Coppel RL, McConville MJ, Eric Maréchal, McFadden GI. "*Lipidomic analysis and functional study of the lipid biosynthesis of the malaria apicoplast"*
- Invited Seminar & symposium organiser, XIIIth Int Congress of Protistology, August 2009 Rio de Janeiro Brazil, "*Apicoplast structure & function"*(speaker: Prof McFadden)
- Invited Speaker/External Expert, Mephitis Malaria Parasite Protein Translation Research Consortium EU/FP7, Delhi India 2 April 2010, "*Lipid and proteome analyses of the Plasmodium falciparum apicoplast"*(speaker: Prof McFadden)
- Invited Speaker, Evimalar Cluster II meeting, Instituto de Medicina Molecular, Lisboa, Portugal 2010 "*Lipidomics of isolated apicoplasts"*(speaker: Prof McFadden)
- Society for Molecular Biology and Evolution, Kyoto, Japan, July 26-30 2011, *"Evolution of galactolipid synthesis in plants, algae and parasites"* (speaker: Prof McFadden)
- Malaria in Melbourne, Royal Melbourne Hospital Convention Center, Melbourne, Australia, October 3-4 2011, Shears M, **Botté CY**, McFadden GI."*Investigating phosphatidic acid synthesis in Plasmodium falciparum apicoplast*" (speaker: Miss Shears)

Posters in International conferences

-International Symposium for Plant Lipids 2010 11-16 july, Cairns, Australia
Botté CY, Yamaryo-Botté Y, Janouskovec J, Keeling P, Coppel RL, McConville MJ, Maréchal E, McFadden GI. *"Identification of plant-like galactolipids in the apicoplast of Chromera velia, an ancestor of malaria parasites*"
-Metabolomics 2011 27-30 July, Cairns, Australia
Rupasinghe T, MacRae JI, **Botté CY,** Yamaryo-Botté Y, McFadden GI, Dull T, McConville MJ. "*LCMS profiling of intra-erythrocyte stages and intracellular organelles of Plasmodium falciparum"*
- 8th GERLI Lipidomics Conference, INSA-Lyon, France, 26-28 October, 2011
Amiar S, VanDooren G, Cesbron-Delauw MF, McFadden GI, Maréchal E, **Botté CY**. "*Role of lipid biosynthesis in the apicoplast of Apicomplexa: Purification of the apicoplast of Toxoplasma gondii"*
- Alg'n' Chem 2011 - Algae, new resources for Industry? Montpellier, France 7-10 November 2011
Botté CY, Yamaryo-Botté Y, Finazzi G, McFadden GI, Maréchal E. *"Introducing Chromera velia, a novel model of microalga"*

International conferences with contributions to be decided
- Keystone Meetings: Drug discovery in protozoan parasites, Santa Fe, USA, 2012 January 15-20
Botté CY, Shears M, Amiar S, Cesbron-Delauw MF, Maréchal E, McFadden GI. *"A novel phosphatidic acid synthesis pathway in the apicoplast as a potential drug target"*
- Molecular Approaches to Malaria, Lorne, Australia, February 2012
Botté CY, Shears M, Amiar S, Cesbron-Delauw MF, Maréchal E, McFadden GI. *"A novel phosphatidic acid synthesis pathway in the apicoplast as a potential drug target"*
Botté CY, Yamaryo-Botté Y, Rupasinghe T, Shears MJ, Mullin KA, Spurck T, Coppel RL, McConville MJ, Eric Maréchal, McFadden GI. "*Lipidomic analysis and functional study of the lipid biosynthesis of the malaria apicoplast"*

- **Publications (peer reviewed)**

LIST OF SCIENTIFIC (PEER REVIEWED) PUBLICATIONS, STARTING WITH THE MOST IMPORTANT ONES								
NO.	**Title**	**Main author**	**Title of the periodical or the series**	**Number, date or frequency**	**Publisher**	**Place of publication**	**Year of publication**	**Relevant pages**
1	*Plasmodium falciparum apicoplast drugs: targets or off-targets?*	*Cyrille Botté*	*Chemical reviews*	*2011 Oct 25. [Epub ahead of print]*	*ACS publications*	*USA*	*2011*	*Not yet available*
2	*Identification of plant-like galactolipids in the apicoplast of Chromera velia, a photosynthetic Apicomplexa.*	*Cyrille Botté*	*Journal of biological chemistry*	*Aug 26;286(34)*	*American Society for Biochemistry and Molecular Biology.*	*USA*	*2011*	*29893-903*
3	*Chemical inhibitors of monogalactosyldiacylglycerol synthases in Arabidopsis thaliana.*	*Cyrille Botté*	*Nature chemical biology*	*Sep 25;7(11)*	*Nature Publishing group*	*USA*	*2011*	*834-842*
4	*Role of Phosphatidic acid in plant galactolipidsynthesis.*	*Emma Dubots*	*Biochimie*	*September 2011 [Epub ahead of print]*	*Elseviewer*	*France/USA*	*2011*	*Not yet available*

Section B (confidential) - EXPLOITABLE FOREGROUND AND PLANS FOR EXPLOITATION

N/A

TABLE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.			
Type of IP Rights: Patents, Trademarks, Registered designs, Utility models, etc.	**Application reference(s) (e.g. EP123456)**	**Subject or title of application**	**Applicant(s) (as on the application)**

Please complete the table hereafter:

TABLE B2: **OVERVIEW TABLE WITH EXPLOITABLE FOREGROUND**					
Exploitable Foreground (description)	**Exploitable product(s) or measure(s)**	**Sector(s) of application**	**Timetable, commercial use**	**Patents or other IPR exploitation (licences)**	**Owner & Other Beneficiary(s) involved**
1. New superconductive Nb-Ti alloy	*MRI equipment*	*1. Medical 2. Industrial inspection*	*2008 2010*	*A materials patent is planned for 2006*	*Beneficiary X (owner) Beneficiary Y, Beneficiary Z, Poss. licensing to equipment manuf. ABC*

In addition to the table, please provide a text to explain the exploitable foreground
[One text box per row in table B2]

N/A

1. SCIENTIST IN CHARGE QUESTIONNAIRE

RESEARCH TRAINING ASSESSMENT:

What is the size of the hosting research group?	10 collaborators (5 permanent researchers, 1 permanent technician, 2 pot-docs, 1 PhD student, 1 non-permanent technician)

How many researchers have you supervised, within the past 10 years? 5 Of which funded by:	
EC/Marie Curie actions	1
EC Other Funding	
University fellowships	
National public bodies	4
Industry	
Other	
Other, please specify:	

How many researchers have you supervised within this project?	1
Corresponding to how many person months?	12

Number of publications resulting directly from the research project: 4	
Recruited researcher(s) and yourself	4
Recruited researcher(s) alone	0
Recruited researcher(s) with authors other than yourself	0

Participation of the recruited researcher(s) at conferences (number): 3	
Passive	
Active	3
How do you rate the overall success of the research training?	Excellent

General assessment: Full success of the programme, with strong assertiveness of the recruited post-doc, indicating that he has become a major scientist of the field. Excellent publication level, with two additional publication under submission, not listed in this table.

RESEARCHERS ASSESSMENT:

Rate the overall level of the recruited researcher(s) integration in the research team and the host organisation with regards to:	
participation in meetings/seminars	Very good
discussions of results and project-related topics	Excellent
co-operation with other team members	Very good
co-operation with other researchers of the host institution	Very good

Rate the overall performance of the recruited researcher(s) with regard to:	
originality of fellow(s) approach towards research (initiative/independent thinking)	Excellent
capacity to develop new skills and to benefit from training	Excellent
productivity (research results/publications/international conference attendance)	Excellent
communication skills	Excellent
group leader skills (collaboration with other groups/project management)	Very good
training and/or teaching skills	Very good
Please comment: The recruited fellow has shown his complete enthusiasm for his project, his ability to be autonomous for the management and steering of his project, his productivity from experiments to publication design and writing. He has also been exceedingly efficient in teaching his techniques to others, including students and researchers, and convincing others to collaborate with him. As such, the recruited fellow has strong leadership potential.	

RESEARCH TRAINING OUTCOMES:

Has this project provided additional links with other research groups or institutions?	Yes
If yes, indicate the number of contacts in each case	4
Universities	4
Research Centres	
Industry/private companies	
Others	
If Other, please specify:	

Rate the importance of the following outcomes of the research training:	
results of the research	Excellent
number of publications	4
development of research	Excellent
establishment of international collaborations	Good
transfer of knowledge/technology	Very Good
training of researcher	Very Good
further academic qualifications (PhD, habilitation etc.) for fellows	Does not apply directly
Please comment:	

YOUR OPINION ABOUT THE MARIE CURIE ACTIONS:

Do you have any other comments or suggestions of how to improve the concerned Marie Curie actions? -

Did you have previous knowledge of the Marie Curie actions?	Yes

If yes, what sort of image do you think that the Marie Curie actions have among the scientific community in your research area?	Excellent and prestigious

Attachments:	

Date: 12 October 2011 **Signature Scientist in Charge:** E. Maréchal 	**Date: 28/11/11** **Signature Researcher:**