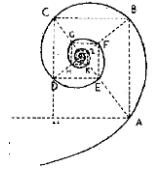**UNIVERSITÀ DEGLI STUDI DI MILANO**

CICLO XXX

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TESI DI DOTTORATO DI RICERCA

MED09

Characterization of the functional role of new variants involved in variegate porphyria and hiPSC derived hepatocyte like cells to model hepatic porphyrias

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RIASSUNTO

Le porfirie sono un gruppo di malattie metaboliche ereditabili legate alla biosintesi dell'eme. Ogni tipo di porfiria è caratterizzata da un peculiare accumulo di precursori dell'eme generato da una specifica alterazione della via biosintetica. Queste malattie rare sono caratterizzate da un'estensiva eterogeneità di mutazioni a carico della regione codificante dei geni direttamente o indirettamente coinvolti nel pathway.

In questo studio, abbiamo valutato tre nuove varianti identificate nelle regioni regolatorie del gene PPOX utilizzando linee cellulari di epatocarcinoma (Hep3B) e di leucemia eritroide (K562). Abbiamo dimostrato una minore espressione del gene PPOX attraverso saggi di luciferasi e analisi del RNA legata alla variante c.1-883G>C, localizzata sul promotore del gene, e abbiamo suggerito un ruolo post-trascrizionale per le varianti c.1-413G>T e c.1-176 G>A site nel 5'UTR della variante 2 del mRNA del gene PPOX. Esperimenti di transfezione del plasmide mutante reporter -413T, indicano che questa variante inibisce la traduzione del mRNA della luciferasi della firefly associata al vettore. In effetti, la ridotta attività della luciferasi della firefly non è correlata con una proporzionale riduzione dell'espressione del mRNA del reporter. I valori normali dei livelli del mRNA del gene PPOX nel paziente portatore della sostituzione c.1-413G>T, validati tramite Digital PCR, supportano questa evidenza. I dati per la variante c.1-176 G>A, mostrano un suo possibile ruolo a livello di regolazione dello splicing. L'analisi qualitativa del RNA conferma che questa mutazione è coinvolta nella soppressione del normale splicing tra l'esone 1 e l'esone 2 dovuto a una delezione di 4 bp nell'esone 1. La relazione tra queste alterazioni post-trascrizionali e la porfiria variegata devono essere ulteriormente investigate. Questi risultati suggeriscono di includere nel processo diagnostico anche le regioni regolatorie dei geni, per cui ulteriori studi sono richiesti per chiarire il loro ruolo nella malattia.

Per creare un modello per le porfirie epatiche, abbiamo creato delle cellule simili agli epatociti a partire dalle cellule pluripotenti indotte generate dalle cellule del sangue di due pazienti affetti da porfiria acuta intermittente, un tipo di porfiria epatica.

Abbiamo ipotizzato che questo potesse servire come un modello in vitro per studiare i diversi pathway legati alla porfiria acuta intermittente.

I dati suggeriscono un profilo generale comparabile della via biosintetica dell'eme tra cellule simili agli epatociti derivanti dalle cellule pluripotenti indotte e gli epatociti primari. Nonostante la permanenza di alcuni caratteri di immaturità, legati ai limiti della riproduzione in vitro degli epatociti, che potrebbero direttamente o indirettamente alterare il network metabolico legato alle porfirie epatiche, al momento le cellule simili agli epatociti derivanti da cellule pluripotenti indotte sono tra i migliori modelli per studiare le malattie epatiche.

Le cellule simili agli epatociti derivanti dalle linee cellulari dei pazienti hanno indicato una maggiore espressione basale del gene ALAS1 e dei suoi regolatori PPARA e PGC1A accompagnata dall'induzione di geni coinvolti nella risposta al danno ossidativo. Inoltre, l'induzione di geni correlati alla chetogenesi e al metabolismo dei lipidi mette in luce un differente profilo metabolico delle linee cellulari dei pazienti affetti da porfiria acuta intermittente.

La simulazione in vitro di un attacco acuto mediante trattamento con ALA, uno dei principali composti accumulati durante l'attacco acuto, ha mostrato una regolazione

negativa sull'espressione dell'ALAS1 e una massiva induzione dell'HMOX1 nella linea cellulare differenziata controllo. Dall'altra parte, la risposta al Fenobarnital nelle cellule di controllo suggerisce il legame della sintesi del colesterolo e della gluconeogenesi con la via biosintetica dell'eme. Il metabolismo del farmaco infatti, induce il pathway biosintetico dell'eme attraverso ALAS1, e questo gene risulta costantemente overespresso nelle linee cellulari dei pazienti.

In conclusione, questo studio focalizza l'attenzione sull'importanza delle regioni regolatorie nei processi diagnostici della porfiria e fornisce un modello in vitro alternativo per studiare le alterazioni metaboliche legate alle porfirie. Ulteriori esperimenti sono necessari per una migliore comprensione degli effetti diretti delle alterazioni nelle regioni regolatorie dei geni legati alle porfirie, e sono richiesti per analizzare il coinvolgimento delle differenti vie metaboliche concorrenti all'espressione della malattia.

ABSTRACT

Porphyrias are a group of inherited metabolic disorders of heme biosynthesis. Each porphyria derives from an alteration in the heme biosynthetic pathway resulting in a specific accumulation of heme precursors. These rare diseases are characterized by an extended heterogeneity of mutations affecting the coding region of the genes directly or indirectly involved in the pathway. In this study, we assessed three new variants identified in the regulatory regions of the PPOX gene using human hepatocarcinoma (Hep3B) and erythroleukemia (K562) cell lines. We demonstrated a lower expression of the PPOX gene through luciferase assays and RNA analysis for the c.1-883G>C promoter mutant and we suggested a post-transcriptional role for the c.1-413G>T and c.1-176G>A variants in the 5' UTR of PPOX mRNA variant 2. Transfection experiments of mutant -413T reporter plasmid indicate that this variant inhibits translation of the downstream firefly luciferase mRNA. In fact, the reduced firefly luciferase activity did not correlate with the proportional reduction in firefly luciferase mRNA expression. Normal values of PPOX mRNA level validated with Digital PCR in the patient carrying the c.1-413G>T substitution support this evidence. Data for the c.1-176G>A variant show a possible role in the spicing regulation for it. The qualitative RNA analysis confirms that this variant is involved in the alteration of the normal splicing between exon 1 and exon 2 of PPOX due to a 4 bp deletions in exon 1. The relation between these post-transcriptional alterations and the variegate porphyria remains to be investigated. These results suggest that the regulatory regions have to be considered in the diagnostic process but more studies are required to clarify their role in the disease.

To model hepatic porphyrias, we derived hepatocyte-like cells from hiPSC generated from blood of two patients affected by acute intermittent porphyria, a hepatic porphyria. We hypothesized that this can serve as an in vitro model to study different pathways linked with acute intermittent porphyria.

The data suggest a general comparable profile of the heme biosynthec pathway between hiPSC-HLC and primary hepatocytes. Although some immature features, probably linked with the in vitro condition, could directly or indirectly affect the metabolism network links with hepatic porphyrias, now hiPSC-HLCs are one of the most useful model available for hepatic diseases.

The patient derived hepatocyte-like cells showed a basal overexpression of ALAS1 and its regulator PPARA and PGC1A with induction of oxidative damage response genes. Moreover, the induction of ketogenetic and lipid metabolic genes highlighted a different metabolic profile in the acute intermittent porphyria patient lines.

The in vitro simulation of acute attack mediated by ALA administration, one of the principal compound accumulated during the attack, showed a negative feedback regulation on ALAS1 with massive induction of HMOX1 in the control cell line.

On the other hand, phenobarbital response in control line suggested the link between cholesterol synthesis and gluconeogenesis with the heme biosynthetic pathway. The drug metabolism in fact, induces the heme biosynthetic pathway through ALAS1, and this gene results constantly overexpressed in the derived patient cell lines.

In conclusion, this study focus the attention on the importance of the regulatory regions in the diagnostic process of porphyrias and supply an alternative in vitro model to study the metabolic alterations linked with porphyrias. Further experiments are required to better understand the direct effect of alteration in the regulatory

regions of porphyria genes and are necessary to analyse the involvement of different pathways in the onset of the disease.

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INTRODUCTION

1.1 Porphyrias

Porphyrias are a group of metabolic disorders due to the accumulation of porphyrin precursors and porphyrins results from the impairment in the heme biosynthetic pathway. Each is caused by a partial or almost total deficiency in one of the enzyme involved in the pathway combining to bring about chemical intermediates peculiar for each form of seven known types of porphyria. The cytotoxic porphyrinogens, with chemical and photoreactive properties, are responsible of the clinical features of the disease such as neurovisceral symptoms and cutaneous lesions. Despite the co-presence in some form of porphyria of neurologic manifestation and photosensitivity, porphyrias can be clinically classified in acute and cutaneous[1].

Acute porphyrias - acute intermittent porphyria (AIP), variegate porphyria (VP) and hereditary coproporphyria (HCP) - present acute attacks due to the accumulation of 5-aminolevulinic acid (ALA) and/or porphobilinogen (PBG) that are markedly detectable in the urine during the attack. In spite of autosomal dominant inheritance of the disease, acute porphyrias are often misdiagnosed for the non-specific symptoms such as abdominal pain, vomiting, nausea, constipation, hypertension, and tachycardia. Moreover, the low penetrance of the disease hides latent patients that could remain asymptomatic throughout life. The neurological symptoms derive from the direct and indirect effects of the overproduced porphyrin precursors ALA and PBG. It has been suggested an alteration of the metabolic state of the cells due to heme deficiency affecting hemeproteins like tryptophan dioxygenase, leads to an increased serotonergic activity. In addition, a malfunction of another hemeproteins, the cytochromes P450, may alter neuronal transmission and the detoxifying capacity of the cell. It has been proposed also a direct neurotoxic effect of ALA acting on the GABAergic system as partial GABA agonist inducing the central nervous system dysfunction. Patients will often also have peripheral neuropathy but the mechanism of the

damage is not well understood[2]. The identification of carriers of the diseases is important to avoid the exposure to porphyrinogenic drugs that, with other events including hormonal changing related to the menstrual cycle, diet, alcohol and stress, could trigger the acute attacks. Porphyric attacks occur mainly after the puberty and affect predominantly women[3].

On the other hand, the skin lesions present in cutaneous porphyria result from the accumulation of reactive porphyrins where the presence of the tetrapyrrolic ring in a photoreactive form produces damage that promotes loss of membrane integrity and oxidation of cellular components.

The feature of the porphyrin structure is its photoreactive to radiation energy in the visible range. By absorbing about 400 nm, porphyrins are excited to a singlet state and they relay the energy absorbed to biomolecules dangerous after oxidation, promoting cellular damage and inflammation. The immunohistochemical analysis of the patient skin suggests the blood vessel of the dermis as principal site of photo injury. The clinical symptoms of the different cutaneous form are linked with the physiochemical properties of the accumulated porphyrins that can affect cytosolic target in case of enzymatic blocks of uroporphyrinogen III synthase (UROS), uroporphyrinogen decarboxylase (UROD), coproporphyrinogen oxidase (CPOX) and protoporphyrinogen oxidase (PPOX) or lipid-rich cellular structure due to ferrochelatase (FECH) deficiency[4]. Lesions are restricted to sun-exposed areas and hyperpigmentation, hypertrichosis and skin fragility are features of the cutaneous poprhyrias. The excretion profile of urinary and faecal porphyrins with also the concentrations in erythrocytes of free protoporphyrin and the plasma porphyrin fluorescence assay are useful parameters in the specific diagnosis of porphyria (table 1).

Poprhyria	Deficient enzyme	Inheritance	Principal symptoms	Biochemical markers			
				Urine	Stool	Red blood cells	Plasma peak (nm)
X-linked protoporphyria	ALAS 2	X-linked	Cutaneous			Free-proto IX	630-634
ALA dehydratase porphyria	ALAD	Autosomal recessive	Neurovisceral	ALA, copro III		Zn-proto IX	
Acute intermittent porphyria	PBGD	Autosomal dominant	Neurovisceral	ALA, PBG, uroporphyrin			618-620
Congenital erythropoietic porphyria	UROS	Autosomal recessive	Cutaneous	Uro I, copro I	Copro I	Uro I, copro I	615-618
Porphyria cutanea tarda	UROD	Sporadic or autosomal dominant	Cutaneous	Uro I/Uro III, hepta	Isocopro, hepta		618-620
Hepatoerythro poietc porphyria	UROD	Autosomal recessive	Cutaneous	Uro III, hepta	Isocopro, hepta	Zn-proto IX	618-620
Hereditary coproporphyri a	CPOX	Autosomal dominant	Neurovisceral and cutaneous	ALA, PBG, coproporphyrin III	Copro III>copro I		618-620
Variegate porphyria	PPOX	Autosomal dominant	Neurovisceral and cutaneous	ALA, PBG, copro III	Copro III, proto IX		624-627
Erythropoietic protoporphyria	FECH	Autosomal recessive	Cutaneous		Proto IX	Free-proto IX	630-634

Table 1: Porphyrias: clinical and biochemical features. Porphyrias can be classified according to the clinical symptoms in neurovisceral and cutaneous. Each porphyria present a peculiar biochemical pattern, important in the diagnosis of the disease.

Considering the site of production and accumulation of porphyrins, porphyria can be also classified as either erythroid or hepatic porphyria. The erythropoietic porphyrias are characterized by overproduction and accumulation of pathway intermediates in bone marrow, whereas in hepatic porphyrias, the overproduction and accumulation of ALA, PBG and porphyrins occur in the liver. In light of this classification there are four acute hepatic porphyrias (acute intermittent porphyria, hereditary coproporphyria, variegate porphyria and ALA-dehydratase porphyria), a single hepatic cutaneous porphyria (porphyria cutanea tarda) and three erythropoietic

cutaneous porphyria (X-linked protoporphyria, congenital erythropoietic porphyria and erythropoietic protoporphyria)[5].

1.1.1 Acute intermittent porphyria

Acute intermittent porphyria (AIP) is the most frequent hepatic porphyria and results from a deficiency of the porphobilinogen deaminase (PBGD) enzyme encoded by the *hydroximetylbilane synthase* (*HMBS*) gene. It is an autosomal dominant disease with low penetrance manifestation. The acute attack generated by different endogenous and exogenous factors are biochemically marked by the urinary overexcretion of ALA and PBG and a plasmatic peak at 618-620 nm. The neurovisceral symptoms are usually intermittent and reversible and affecting principally the women after puberty[6].

The *HMBS* gene is located at chromosome 11q24.1–24.2[7] and is composed by 15 exons. Alternative splicing transcripts are generate from the same gene for the erythroid specific and ubiquitous isozymes. The housekeeping promoter is located upstream the exon 1 while the erythroid promoter, recognized by erythroid specific transcription factors, is in the intron 1. The erythroid enzyme differs in the amino-terminal sequence part 17 additional amino acid residues contained in the ubiquitous enzyme[8].

The use of mouse model has been used to study the mitochondrial energetic failure and glucose metabolism linked with acute intermittent porphyria[9]. At now, only one study was performed to study the biochemical and pathologic alteration in the liver of a transplanted AIP patient[10].

1.1.2 Variegate porphyria and PPOX gene

Variegate porphyria is a low penetrance autosomal dominant hepatic porphyria due to enzymatic defect of protoporphyrinogen oxidase. The acute attacks are associated with urinary excretions of ALA, PBG uro- and

coproporphyrins combined with a plasmatic peak at 624-627 nm. The neurovisceral symptoms may occur with photosensitivity[6].

The *PPOX* is transcribed by the gene localized in the 1q22.23 region[11]. A single promoter controls the housekeeping and erythroid transcription. Tissue specific variants producing the same functional protein have been isolated. The shorter variant 1 has been isolated in different tissues such as liver, heart, pancreas, brain, placenta, lung, kidney and skeletal muscle[12]. The variant 2 was predominant in leukocytes.

The promoter region contains CCAAT box and Sp1 binding site for the basal transcription activity localized in the upstream part of the promoter[13], whereas the erythroid transcription is supported by GATA-1 binding sites in the 5'UTR region[14] but the tissue specific and induced transcription remain unknown. The gene is composed by 13 exons where the exon 1 and a section of exon 2 are part of the 5'UTR of the gene. The starting of translation (ATG) is located in the exon 2[13].

1.2 Heme biosynthetic pathway

This is one of the most conserved pathways between all the species leads the formation of a tetrapyrrolic structure associated to different metals constitutive of the "pigments of life". The heme in particular, is composed by a Fe^{2+} ion conjugated with the protoporphyrin IX produced by the sequential action of eight enzymes located between the mitochondria and cytosol of all the cells[15]. The synthesis of the heme starts in the mitochondria from the condensation of succinyl Co A and glycine by the enzyme ALA synthase (ALAS) to form 5-aminolevulinic acid (ALA) (fig. 1).
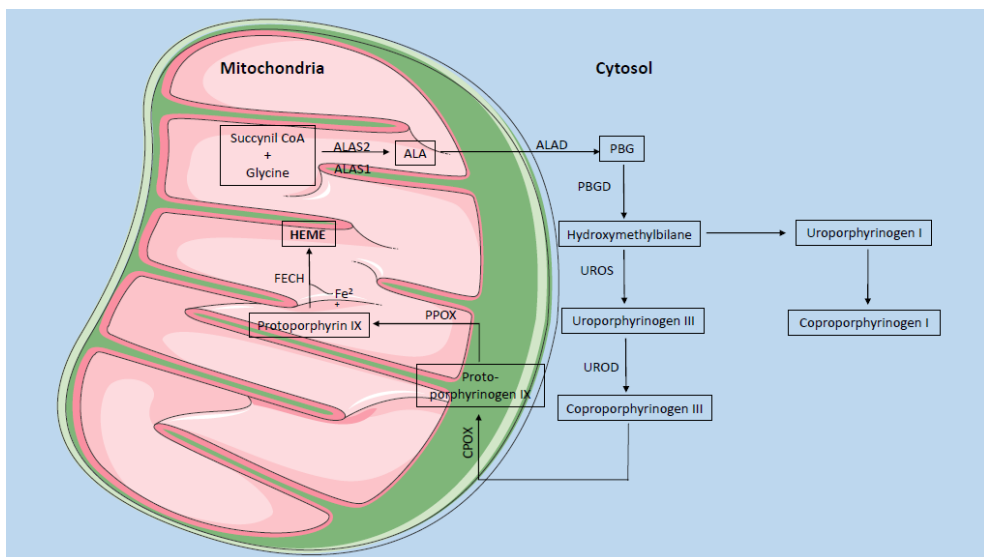
Fig 1 Heme biosynthetic pathway. From succinyl CoA and glycine through the action of the eight enzyme of the pathway between mitochondria and cytosol there is the formation of the tetrapyrrolic ring conjugated with Fe^{2+}, heme.

The human ALAS is coded by two different genes, one for the housekeeping form, *ALAS1* on chromosome 3, and one for the erythroid form, *ALAS2* on chromosome X. The condensation of two molecules of ALA in one molecule of porphobilinogen (PBG) is made by ALA dehydratase (ALAD) and takes place in the cytosol. Subsequent to the link of four molecules of PBG by porphobilinogen deaminase, the hydroxymethylbilane is cyclized in uroporphyrinogen III by uroporphyrinogen III synthase (UROS). In the absence of UROS, the hydroxymetilbilane can spontaneously cyclizes to form first the uroporphyrinogen I and after decarboxylation, the isomeric coproporphyrinogen I. The uroporphyrinogen decarboxylase (UROD) catalyzes the sequential decarboxylation of four carboxylic group of the acetic-acid side to form coproporphyrinogen III. After the transport into mitochondria, the coproporphyrinogen oxidase (CPOX) catalyzed the formation of vinyl groups by the removal of two of the four propionate groups. Then, the protoporphyrinogen IX is oxidized in protoporphyrin IX by

protoporphyrinogen oxidase (PPOX). The terminal step of the pathway is catalysed by the ferrochelatase (FECH) responsible of the insertion of the iron into the tetrapyrrolic ring to form the heme[16].

The heme is the main component of the hemeproteins involved principally in the transport and storage of oxygen, the sterols, lipids and neurotransmitters metabolism, oxidative stress reaction, control and generation of the energetic metabolism of the cells[17]. Although heme is produced in all the cells for the essential respiratory and energetic reactions, it is mainly synthetized in the bone marrow to for haemoglobin during erythroid differentiation (80% of the synthesis) and in the liver (15% of the synthesis) as constituent of cytochromes.

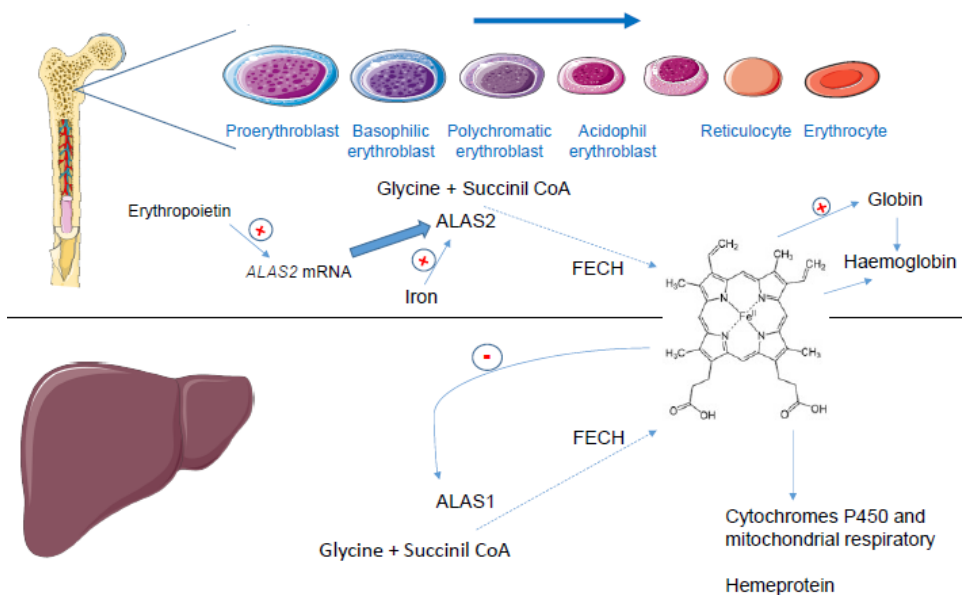**Fig 2 Differential control of heme biosynthetic pathway in bone marrow and liver.** During hematopoiesis, *ALAS2* is active for the production of heme involved in the haemoglobin formation. It is induced by erythropoietin and iron. In the liver *ALAS1* is recruited for supplying of the heme for different hemeprotein, cytochromes involved in drug metabolisation and respiratory chain. *ALAS1* is negatively regulated by heme.

The two tissues differ principally on the control on the first rate limiting enzyme of the pathway, *ALAS 2* for the erythroid cells and *ALAS 1* for the liver and all the other cells and reflect also the differential request of heme between the organs (fig 2).

ALAS 2 has to support the constant production of haemoglobin and it is induced by erythropoietin in response to hypoxia signal. Thereafter, induction of transcriptional factors as *GATA1*, *CACC box* and *NFE2* activates the expression of *ALAS2* that is post-transcriptionally controlled by the availability of iron in the cell. In iron deficiency in fact, the IRE-binding proteins bind the IRE sequence located in the 5'untranslated region of *ALAS2* and prevent the accumulation of toxic protoporphyrins[18]. The impairment of activity of ALAS2 causes X-linked sideroblastic anemia for iron overload due to insufficient synthesis of protoporphyrins, or X-linked erythropoietic porphyria as result of ALAS2 activity increase[19].

In the other cells and especially in the liver, *ALAS1* has to support the rapid metabolic changes requiring hemeproteins. This rate-limiting step is regulated through negative feedback by the free heme pool. The inhibitory action of heme on *ALAS1* occurs at different levels. Together with a repressive action on transcription and degradation of mRNA, heme prevents the mitochondrial translocation of ALAS1. On the other hands, the upregulation of *ALAS1* is mediated indirectly by HMOX1 through the catabolism of heme. Additionally, transcriptional factor as *PGC1α* and *PPARα* contribute to *ALAS1* induction and participated to the precipitation of acute attacks linked with drugs, hormonal and glucose metabolism[20]. Considering the central role as pathogenetic triggering actor in acute porphyria patients, hepatic *ALAS1* is the target of a phase I clinical trial with a RNAi conjugated with GalNAc, a ligand for a liver-specific receptor, to reduce the activity of the enzyme and the accumulation of the toxic porphyrins[21].

1.3 Heme catabolism

To prevent the toxic accumulation of compound derived by production of porphyrin precursors and porphyrins, the cells are able to handle heme leftover from the breakdown of heme proteins by the heme catabolism.

Heme oxigenase 1 (HMOX1) is a protein ubiquitously expressed but is principally induced in liver and spleen, where the main catabolism of haemoglobin takes place.

The enzyme cleaves one of the methane bridges of the porphyrin ring in concert with NADPH cytochrome reductase with generation of carbon monoxide. Then, the linear tetrapyrrol biliverdin, is oxidized to bilirubin with release of available iron for the cell.

HMOX1 is induced by different stimuli as heavy metals, oxidative stress organic chemicals and heme by its self. Stimuli that increase *HMOX1* may thereby trigger clinical symptoms in some forms of porphyria by overload of the deficient enzyme due to the lack of heme free pool on the pathway dependent on HMOX1 activity[4].

1.4 Human induced pluripotent stem cells (hiPSCs)

The human induced pluripotent stem cells (hiPSCs) are reprogrammed somatic cells that after the introduction of four crucial genes, *octamer-binding transcription factor 4* (*OCT4*), *sex determining region Y* (*SRY*)-*box 2* (*SOX2*), *krüppel-like factor 4* (*KLF4*) and *cellular homolog of v-myc avian myelocytomatosis viral related oncogene* (*c-Myc*), have all the pluripotency characteristics as the self-renewal and the capacity differentiation toward all the three somatic germ layers: ectoderm, mesoderm and endoderm[22].

The possibility to generate expansible and tissue specific differentiated cells from somatic cells as fibroblast or blood cells, opened the opportunity to multiple applications such as disease modelling, drugs screening and autologous transplantation. The capacity of these cells to differentiate in any tissue, offers the opportunity to create *in vitro* a disease modelling maintaining the genetic background of the patients and reproducing the phenotype of the original donor. Their potential will permit in the future the use of these cells for individual drug screening or gene therapy application. Different methods have been proposed for the delivery of the reprogramming factors including viral vectors integration in the DNA of host cells or by reproducing of the vector restricted in the cytoplasm. Other methods as small molecules and episomal vector are recently used for the transgene-free human reprogramming[23] .
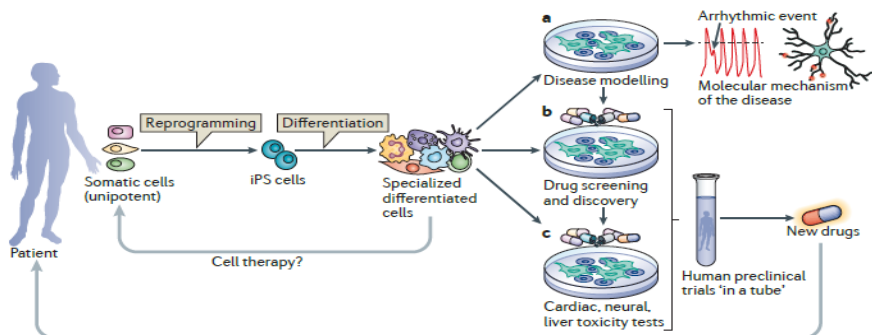

Milena Bellin et al *Nature Reviews Molecular Cell Biology* 2012

1.5 Stem cells derived hepatocytes

The capacity of pluripotent stem cells to generate all cell type of the human body makes them the ideal cell type to establish disease modelling on tissues that are hard to study for the limited source of primary tissues. The liver is metabolic hub of the body interacting with different tissues as gastrointestinal tract, skeletal muscle and adipose tissue by crosstalk with the neuronal and hormonal systems. The liver is a complex organ composed by different cell types with specific functions. The hepatocytes are the major cell type and participate to the principal liver functions. They control the body energy metabolism by glucose and ammonia homeostasis, fatty acid biosynthesis and degradation, cholesterol and glycogen synthesis and they express detoxification capacity mediated by CYPs drug metabolisation activity. Several transcription factors and coactivators control liver energy metabolism and make the important metabolic switch in the fed or fasted states to store or synthetize glucose under the tightly control of insulin and other metabolic hormones[24].

The protocol of hepatic differentiation has the goal to produce efficiently and in a reproducible manner cells with phenotypic and metabolic feature of primary hepatocytes.

Functionally, the primary hepatocytes express mature markers as albumin, transthyretin (TTR) and urea synthesis. Moreover, they presents high expression of drug metabolizing enzymes, cytochromes P450s, and transporter proteins as NTCP, important for hepatothropic viral infection. Expression profile comparison between fresh human hepatocytes and stem cells derived hepatocytes showed a fetal hepatic phenotype for the

hepatocyte-like cells derived from stem cells[25]. Many studies suggest an improvement of the in vitro culture conditions to create mature hepatocytes. The primary hepatocytes remain the gold standard cell type for application as drug metabolisation studies[26]. However the limited supply resource of donors and the quick dedifferentiation of primary hepatocytes in the *in vitro* culture conditions[26], limit their application on modelling disease supporting the use of stem cells derived hepatocytes.

To differentiate hiPSCs toward hepatocytes, the initial endodermal commitment is mediate by Activin A and Wnt3A. The hepatoblast differentiation is driven by bone morphogenetic protein 4 (BMP4) and fibroblast growth factors 1 (FGF1) and 4 (FGF4) though hepatic endodermal specification. The final maturation step is promoted by hepatic growth factor (HGF).

AIM

In the first part of this PhD project, we aimed to evaluate the functional role of nucleotide variants identified in variegate porphyria patients without other mutations in the coding region of the *PPOX* gene.

To this end, we formulated the following objectives:

- Expression assessment of the *PPOX* gene in the patients
- Generation of reporter plasmid vector containing the mutated region of interest
- Luciferase assay
- RNA analysis

In the second part of the PhD project, we aimed to generate human induced pluripotent stem cells to model hepatic porphyria.

For this part of the project the objectives were:

- Generate human induced pluripotent stem cells from blood samples of the patients with high quality standard value
- Differentiate the hiPSCs into hepatocyte, and value the capacity of the AIP patients lines to follow the differentiation program
- Metabolic characterization of the differentiate hepatocyte derived from AIP patients
- Expression analysis of pathways related with AIP phenotype
- Reproduction in vitro of acute attack by porphyrinogenic compounds and drugs treatment

MATERIALS AND METHODS

3.1 Patients

Five unrelated patients with symptomatic and clinical indication of variegate porphyria and two unrelated patients with molecular diagnosis of acute intermittent porphyria were recruited. Peripheral blood samples were collected in accordance with ethical principles and after signature of the informed consent approved by ethics committees of the Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico di Milan and KU Leuven.

3.2 Plasmid construction and luciferase assays

The plasmid construction and luciferase assay is described in the paper[27]. Briefly, the region with the promoter and 5'UTR of the *PPOX* gene was isolated by polymerase chain reaction and TA cloned into pGL4.10 plasmid. To generate promoter plasmids with the mutation of interest, the wild type promoter-pGL4.10 construct was directly mutagenised by QuikChange Site-Direct Mutagenesis Kit (Stratagene, La Jolla, California, USA). To perform the luciferase assay after transient transfection using Fugene Transfection Reagent (Promega Corp. Madison USA) of the constructs in presence of Renilla Luciferase-TK vector as efficiency control, the luciferase activity was determined by Dual-Luciferase Report Assay System with GloMax Discovery system (Promega Corp. Madison USA) 48h after transfection. The relative luciferase activity was calculated after normalization of the Firefly on Renilla activity and the data were expressed as a ratio of activity of the mutated promoter/reporter construct and that obtained for wild-type promoter/reporter construct, under the same conditions (FI; fold induction).

3.3 Peripheral Blood Mononuclear Cells extraction

The samples were diluted with PBS (1:1) and extracted by Ficoll separation using Ficoll Paque Premium reagent (GE Healthcare). After centrifuge separation for 5 min at 300xg, the peripheral blood mononuclear layer was

extracted and washed with PBS 3 times. The cells were stored in liquid nitrogen.

3.4 Cell cultures

The human hepatocellular carcinoma cell line (Hep3B) and human erythroleukemia cell line (K562) were cultured as described in [27]

The peripheral blood mononuclear cells were cultured in SFEMII media with L-glutamine (2mM), SCF, FLT3, IL-3 and IL-6.

The reprogrammed cells were initially cultured in peripheral blood mononuclear cells media. After 5 days, the cells were cultured in ReproTeSR Reprogramming medium in presence of Mouse Embryonic Fibroblast. After 20 days they were cultured in E8 flex Medium (Invitrogen) on human qualified Matrigel (BD Bioscience).

The hiPSC were differentiated towards hepatocytes following a previously described protocol with modifications under patent's protection[28].

The cells were incubate at 37°C with 5% of CO_2.

The Sigma cell line used as control are the iPSC epithelial-1 from Sigma-Alderich. To limit epigenetic memory differences between the iPSC derived from different tissues, the differentiation process was induced after several cell division passages.

3.5 Sendai reprogramming

The transduction of $2,5*10^5$ cells was performed with the CytoTune iPS 2.0 Sendai Reprogramming Kit following the manufacturer's procedure (Invitrogen).

3.6 RNA extraction and quantitative reverse transcription PCR

The total RNA was extracted by Maxwell 16 LEV simplyRNA Blood Kit (Promega Corporation) following the manufacturer's procedures. The total RNA was reverse transcribed using high-capacity cDNA archive kit. The PPOX expression was analysed on ABI PRISM 7500 real time PCR system (Thermofisher) using TaqMan gene expression assays in combination with

TaqMan Universal Mater mix. Custom assays for firefly and Renilla luciferase were designed. Primer list and Taq Man assay codes in [27]

The RNA from derived-hepatocytes and primary cell lines was isolated by the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) following manufacturer's procedures. Genomic DNA contamination was degraded by On-Column DNase 1 Digestion kit (Sigma-Aldrich). The Superscript III First-Strand synthesis system (Invitrogen) was used for the cDNA synthesis. The quantitative real-time PCR was performed in a Vii7 Real Time PCR instrument (Thermofisher) with the Platinum Sybr green qPCR supermix-UDG kit (Invitrogen). The data were normalized with the $2^{-\Delta\Delta CT}$ method[29] using the Ribosomal Protein L19 (RPL19) as housekeeping.

3.7 Digital PCR and qualitative RNA analysis

The qualitative RNA analysis and confirmation of PPOX relative gene expression was described in [27]. Briefly, the 25 ng of cDNA was loaded to QuantStudio 3D Digital PCR 20K Chip with 1X of QuantStudio 3D Digital PCR Master mix and 1X of Taqman assay. The housekeeping and target gene expression was determined by independent assays. The ratio of the target on endogenous gene express as copies/μl were compared to value of the subject control. For the qualitative RNA analysis, the region of interest was amplified from cDNA sample and submitted to direct sequencing.

3.8 Intracellular flow cytometry

The intracellular AAT flow cytometry staining was performed on a single cell suspension made by liberase treatment (Roche) and fixed with 4% of PFA. The permeabilisation was mediated by a 0,1% of saponin solution blocked for 45 min with 10% of goat serum (Dako). The cells were stained with 62,5 ng/200 μl/10^6 cells anti-AAT antibody (Dako) or rabbit IgG isotype control (BD Pharmigen) for 1h at room temperature. The secondary Alexa Fluor 647 antibody (1:1500)(Invitrogen) was incubate with cells for 30 min at RT. Cells were analysed by flow cytometry analysis using a FACS-Canto (BD).

3.9 Immunofluorescence

Cells were fixed with 4% of PFA and the permeabilisation with 0,2% Triton X-100 was blocked with 5% donkey serum (Jackson Laboratory). The cells were stained overnight at 4°C with the primary antibodies and isotype controls. The cells were later incubate with the appropriate secondary antibodies together with Hoechst (Sigma-Aldrich) for 60 minutes at room temperature. The images were generated by AxiomagerZ.1 fluorescence microscope.

3.10 Albumin ELISA

The albumin ELISA was performing according to the manufacturer's procedure (Bethyl) on the media collected at D20 of the differentiation protocol. The supernatant was incubate with a primary albumin antibody for 1 h. The HRP- detection antibody was added for 1h and afterward, the TMB-peroxidase solution was added for 15 min. The reaction was stopped by a 0,18M H_2SO_4 solution. The OD was measured on a microplate reader at a wavelength of 450 nm. The amount of albumin was calculated using a generated standard curve.

3.11 CYP assay

The CYP assay was performed using the fluorometric probe 7-benzyloxy-4-trifluoromethylcoumarin (BFC) as described by [30] specific for the CYP3A4 metabolism.

Metabolisation was measured after 1h of incubation times.

3.12 Statistical analysis

The data were analysed by GraphPad Prism software. The statistical analysis was performed by unpaired t test after normal distribution verification through R Shapiro test.

RESULTS

4.1 Characterization of the functional role of new variants involved in variegate porphyria

4.1.1 Identification of new variants in symptomatic patients with variegate porphyria

Five patients with clinical and biochemical features of variegate porphyria (VP) carried three different variants in the promoter and 5'UTR region of the *PPOX* gene (NC_000001.11): c.1-883G>C, c.1-413G>T, c.1-176G>A. Three of them presented the c.1-176G>A variant. These variants are not common polymorphisms in the Single Nucleotide Polymorphysm (dbSNP) and Exome Aggregation Consortium (ExAC) databases. No other alteration or mutations were detected in the coding region of the gene.

4.1.2 PPOX gene expression in the patients

To determine the possible down-regulation of *PPOX* in the patients with the new identified variants, we performed quantitative real-time PCR (qRT-PCR) and digital PCR on the RNA extracted by the peripheral blood of the patients and healthy controls. The results showed a lower expression of the gene compared to two different housekeeping only in the patient with the c.1-883G>C variant (Fig 5).
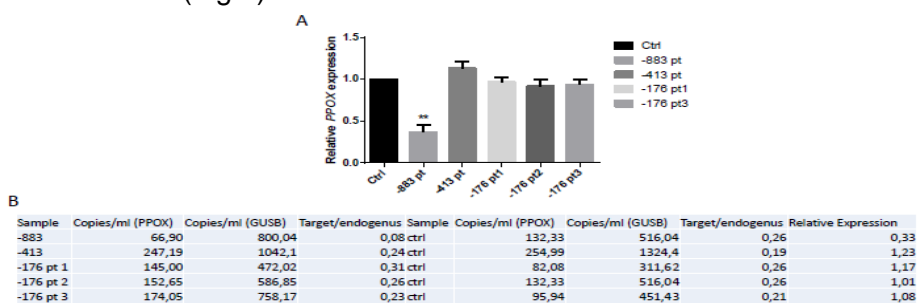

B

Sample	Copies/ml (PPOX)	Copies/ml (GUSB)	Target/endogenus	Sample	Copies/ml (PPOX)	Copies/ml (GUSB)	Target/endogenus	Relative Expression
-883	66,90	800,04	0,08	ctrl	132,33	516,04	0,26	0,33
-413	247,19	1042,1	0,24	ctrl	254,99	1324,4	0,19	1,23
-176 pt 1	145,00	472,02	0,31	ctrl	82,08	311,62	0,26	1,17
-176 pt 2	152,65	586,85	0,26	ctrl	132,33	516,04	0,26	1,01
-176 pt 3	174,05	758,17	0,23	ctrl	95,94	451,43	0,21	1,08

Fig 5 Relative expression of PPOX gene. (A) qRT-PCR of the *PPOX* mRNA detected in the blood of the patients. The relative expression was compared to healthy controls after normalization against two different housekeeping genes. The results are reported as the mean ± SD of three independent experiments. ** indicates p values<0,005. (B) Digital PCR expression assy. The relative expression in the patients results as ratio of the copies/μl of the target gene on the endogenous compared to the healthy control with a confidence level of 95% and precision below 10%. *GUSB* was used as housekeeping gene.

4.1.3 Luciferase assay to characterize the functional role of the variants

To investigate the role of the variants, the region of interest was cloned upstream the reporter gene Luc2 into the pGGL4.10 basic vector (fig 6).
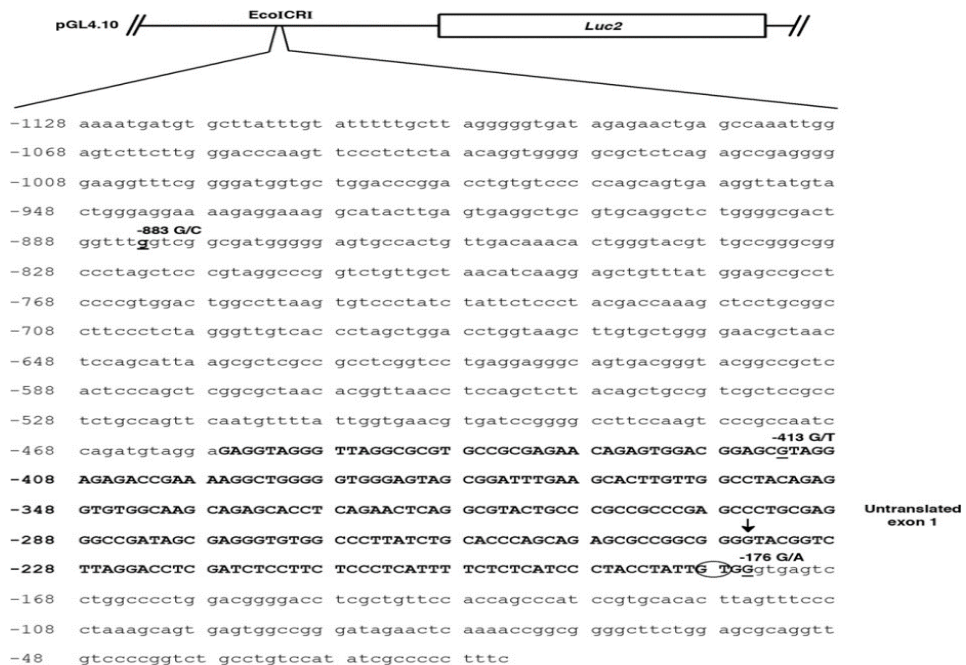


```
                                EcoICRI
pGL4.10 //─────────────────────────────┐         ┌────────────────────┐ //
                                        │         │       Luc2         │
                                        └─────────┘                    └─

-1128  aaaatgatgt gcttatttgt atttttgctt aggggggtgat agagaactga gccaaattgg
-1068  agtcttcttg ggacccaagt tccctctcta acaggtgggg gcgctctcag agccgagggg
-1008  gaaggtttcg gggatggtgc tggacccgga cctgtgtccc ccagcagtga aggttatgta
-948   ctgggaggaa aagaggaaag gcatacttga gtgaggctgc gtgcaggctc tggggcgact
              -883 G/C
-888   ggtttggtcg gcgatggggg agtgccactg ttgacaaaca ctgggtacgt tgccgggcgg
-828   ccctagctcc cgtaggcccg gtctgttgct aacatcaagg agctgtttat ggagccgcct
-768   ccccgtggac tggccttaag tgtccctatc tattctccct acgaccaaag ctcctgcggc
-708   cttccctcta gggttgtcac cctagctgga cctggtaagc ttgtgctggg gaacgctaac
-648   tccagcatta agcgctcgcc gcctcggtcc tgaggagggc agtgacgggt acggccgctc
-588   actcccagct cggcgctaac acggttaacc tccagctctt acagctgccg tcgctccgcc
-528   tctgccagtt caatgtttta ttggtgaacg tgatccgggg ccttccaagt cccgccaatc
                                                                -413 G/T
-468   cagatgtagg aGAGGTAGGG TTAGGCGCGT GCCGCGAGAA CAGAGTGGAC GGAGCGTAGG
-408   AGAGACCGAA AAGGCTGGGG GTGGGAGTAG CGGATTTGAA GCACTTGTTG GCCTACAGAG
-348   GTGTGGCAAG CAGAGCACCT CAGAACTCAG GCGTACTGCC CGCCGCCCGA GCCCTGCGAG   Untranslated exon 1
-288   GGCCGATAGC GAGGGTGTGG CCCTTATCTG CACCCAGCAG AGCGCCGGCG GGGTACGGTC
                                                                -176 G/A
-228   TTAGGACCTC GATCTCCTTC TCCCTCATTT TCTCTCATCC CTACCTATTG TGGgtgagtc
-168   ctggcccctg gacggggacc tcgctgttcc accagcccat ccgtgcacac ttagtttccc
-108   ctaaagcagt gagtggccgg gatagaactc aaaaccggcg gggcttctgg agcgcaggtt
-48    gtccccggtct gcctgtccat atcgcccccc tttc
```

Fig. 6 Plasmid construction. A region of 1114bp including the promoter and 5'UTR region of the PPOX gene was cloned in the pGL4.10 vector upstream the reporter *Luc2*.The c.1-883G>C is located in the promoter, while the c.1-416G>T and c.1-176G>A in the 5'UTR indicated in upper bold case. The arrow indicates the alternative splice site of the variant 1. The circle indicates the alternative splice generated by the c.1-176G>A variant.

The wild type and mutated constructs were transfected in Hep3B and K562 cell lines and the activity of the luciferase was normalised on the Renilla luciferase co-transfected as internal control. The results indicated a significant reduction of the luciferase activity in both cell lines for the -883C and -416T constructs, in contrast with a normal activity for the -176A construct with a more evident effect on Hep3B.

Considering the localisation of the variants in the promoter and 5'UTR of the PPOX gene, we quantified the mRNA level of luciferase in the transfected cells to clarified the level of regulation on the PPOX content due to these variants. The relative mRNA quantification showed a lower expression of luciferase for the -883C construct, proportional with the lower activity of the enzyme. In contrast with a reduced activity of luciferase, the mRNA content of the -413T construct was similar to the wild type construct, as well as for the -176A construct in both cell lines. The data for the -413T construct suggested a translational involvement of the variant more than a transcriptional role (fig 7).
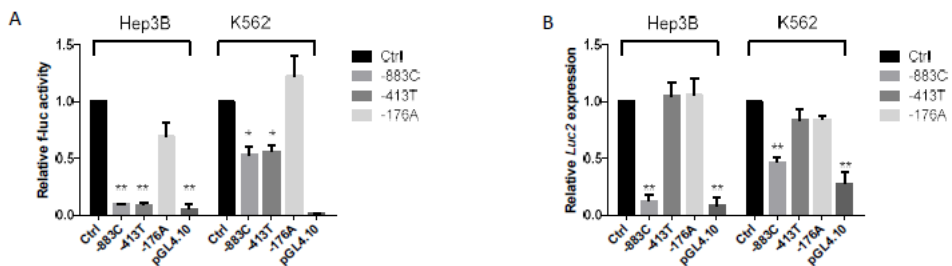
Fig 7 Luciferase assays. (A) Relative firefly luciferase activity in Hep3B and K562 cell lines. The data results from the ratio between Luciferase and Renilla activity compared to the wild type construct (Ctrl). pGL4.10 indicates the empty vector. *=pvalue <0,05; **=pvalue<0,005. (B) Relative *Luc2* expression in the transfected cell lines. The relative expression of *Luc2* was normalized against Renilla mRNA and was compared to the cells transfected with the wild type construct (Ctrl). pGL4.10 indicates the empty vector. *=pvalue <0,05; **=pvalue<0,005. The results are reported as the mean ± SD of three independent experiments.

4.1.4 Qualitative mRNA analysis to test the c.1-176G>A variant

To determine the role of the c.1-176G>A variant, located in the last nucleotide of the 5' untranslated exon 1 of the PPOX gene, we performed the qualitative reverse transcription PCR (RT-PCR) selecting the region between exon 1 and exon 3. No difference were highlighted in the amplification data of the patients compared to the controls on agarose gel verification. The direct sequence of the mRNA, by contrast, indicated a

deletion of 4bp in all patient with the c.1-176G>A variant indicating a splicing modulation role for it by activation of a cryptic splicing site (Fig 8).
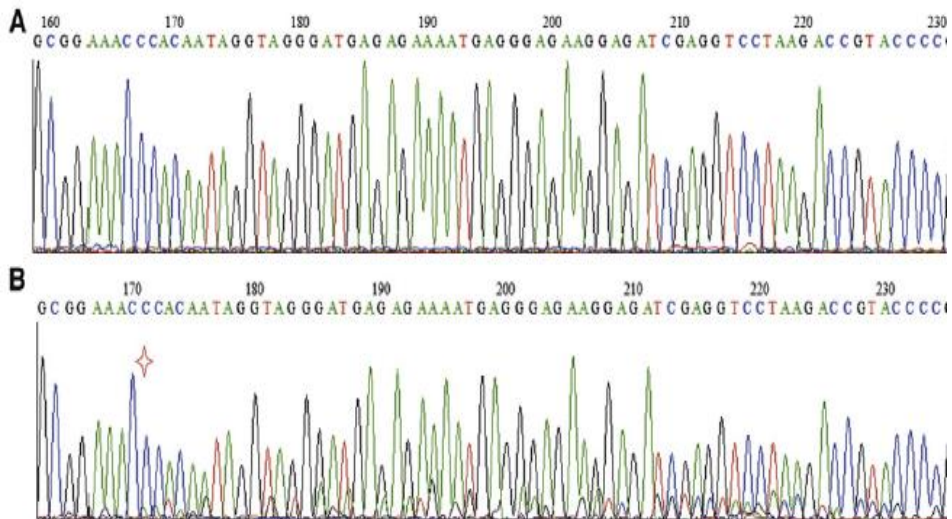

Fig 8 Electropherograms of RNA direct sequencing. The panel A indicates the wild type sequence from a healthy donor. The panel B shows a frameshift due to a 4bp deletion in all the patients carrying the c.-176G>A variant. The red star indicates the beginning of the frameshift.

The mRNA originated by this variant did not showed stability alteration as shown by previous relative and qualitative assay of *PPOX* gene in the patients. The direct effect on the protein level remain unknown.

4.2 HiPSC derived hepatocytes like cells to model hepatic porphyrias

4.2.1 Generation of hiPSC from AIP patient lines

To model hepatic porphyrias we generated two human induced pluripotent stem cell (hiPSC) lines from the peripheral blood of two unrelated symptomatic patients with acute intermittent porphyria (AIP).

To demonstrate the reprogramming of the somatic cells to iPSCs, we tested the absence in the different clones of the Sendai viral vector and the expression of pluripotency markers as *SOX2, OCT4* and *NANOG* through quantitative RT-PCR. The pluripotency was also confirmed with immunofluorescence staining of undifferentiated iPSCs for the pluripotency markers SOX2, OCT4 and NANOG and embryoid body formation assay, followed by ScoreCard® analysis. The single-nucleotide polymorphisms (SNPs) profile indicated no difference between the blood original samples and the generated clones. Moreover, we selected clones without significant genome-wide aberrations on aCGH-array (Fig 9).
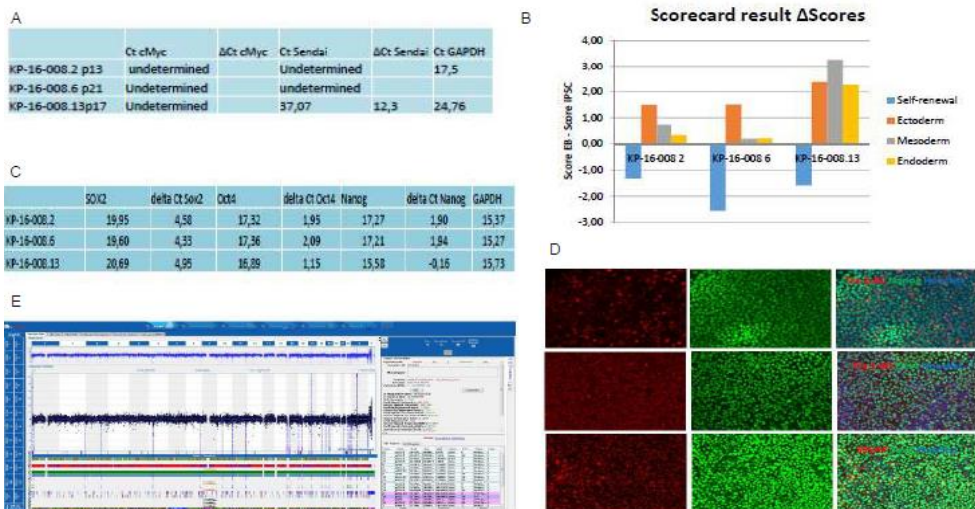
Fig 9 Characterization of hiPSCs from acute intermittent porphyria patients. (A) Expression of cMyc and Sendai virus in the individual clones. (B) Embryoid body formation assay demonstrating differentiation to the three germ layers and loss of pluripotency (C) Expression of pluripotency marker *SOX2*, *OCT4* and *NANOG* compare to *GAPDH*. (D) hiPSC were stained for NANOG, SOX2 and OCT4. (E) Comparative genomic hybridization (CGH) assay. Clones present Derivative Log Ratio (DLR) <0.20.

The presence of the mutation of patient 1 (AIP1) and patient 2 (AIP3) in the generated hiPSC lines was validated with direct sequencing and compared to the Sigma cell line used as control sample (Fig.10)
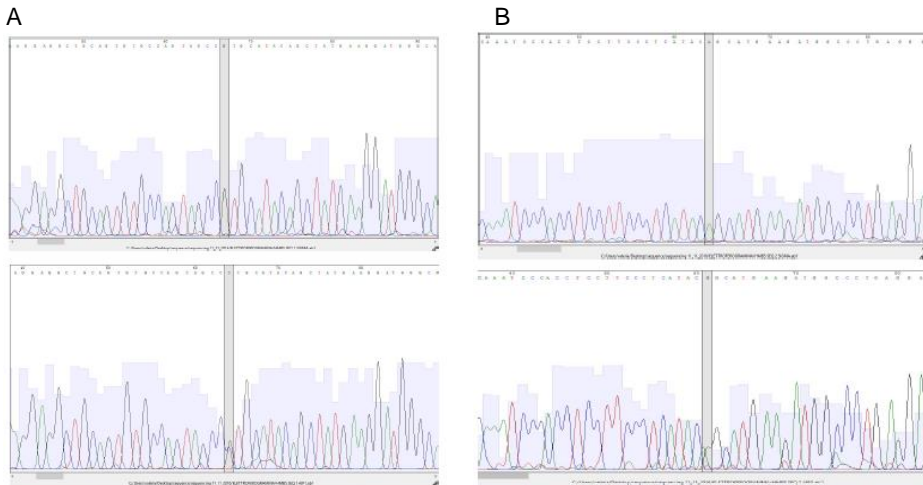

Fig 10. Direct sequencing of hiPSCs from AIP1 and AIP3. (A) The sequencing of Sigma cells (upper panel) showed presence of the wild-type G base in position c.799 of *HMBS* gene (NM_00190.3) while in the AIP1 iPSC line G was replaced by C. (B) Sequencing of Sigma cells in position c.913-2 identified an A while the A was replaced by a G in the AIP3 iPSC line.

4.2.2 Differentiation of hiPSCs in HLCs

The hiPSCs were differentiated toward hepatocytes-like cells (HLCs) using a differentiation protocol of 20 days, adapted from the protocol described in [28]. To validate the HLCs as model for porphyria, we first analysed the expression of genes involved in the heme biosynthetic pathway in the healthy donor Sigma iPSC derived HLCs and primary human hepatocytes (PHHs) from three different healthy donors.

Although the expression analysis demonstrated a comparable profile for the genes of the pathway, there was a significant reduction for *ALAS1* and *CPOX* that might be linked with the immature phenotype of HLCs (Fig.11).

Heme biosynthetic patway in PHH and Sigma

Fig 11. The heme biosynthetic pathway in PHH and Sigma derived HLCs. Expression of the heme biosynthetic pathway was similar between the PHH and Sigma derived HLCs with the exception of *ALAS1* and *CPOX.* The results are reported as mean of the absolute expression for each gene ± SD relative to *RPL19* expression of three independent experiments. PHH represents primary human hepatocytes plated for 24h derived from three unrelated healthy donors. Statistical analysis performed with unpaired t-test after normal distribution verified with R Shapiro.test. *=pvalue<0.05; **=pvalue<0,005 indicate significant difference between the Sigma HLCs and PHH.

The HLCs from the healthy donor Sigma, AIP1 and AIP3 iPSC lines showed the typical cuboidal shape of differentiated HLCs (fig 12) and the flow cytometry analysis on HLCs stained with an antibody against alpha-1-antytrypsin demonstrated that 88-98% stained positive (fig 13).
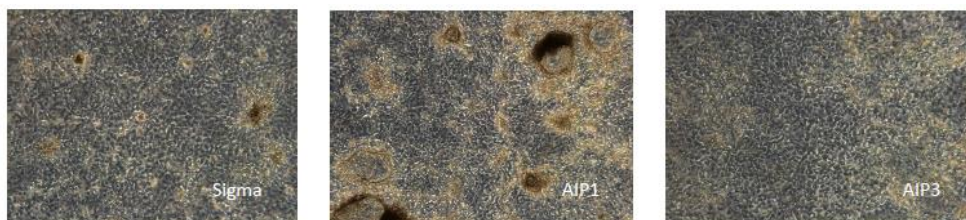
Fig.12 Cuboidal shape of the hiPSC-derived HLCs. Bright-field microscopy images of HLCs at D20. Similar images where produced by 3 independent different experiments.
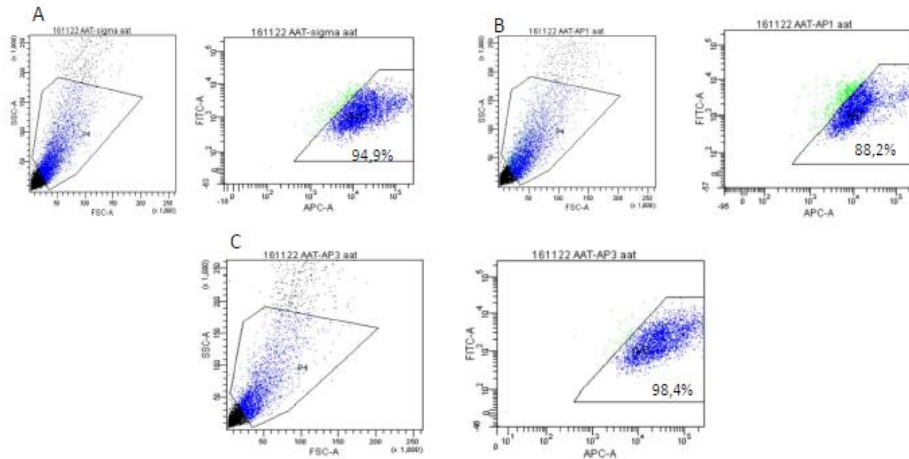
Fig 13 Flow cytometry analysis. FACS demonstrated that approximatively 90% of cells were positive for AAT for Sigma (A), AIP1 (B) and AIP3 (C) HLCs. This analysis is representative of two independent HLCs differentiation experiments for each cell line.

We next performed qRT-PCR to assess expression of a series of marker genes involved in hepatic differentiation and we compared the HLCs-derived lines from the two patients with the HLCs-derived from the Sigma control line. Some transcripts, such as *ALB* and *HNF6,* were lower express in the HLCs derived from the two patient lines, while *HNF4* and *AAT* were comparable to the healthy control line or more highly expressed in AIP3 derived cells (Fig14).
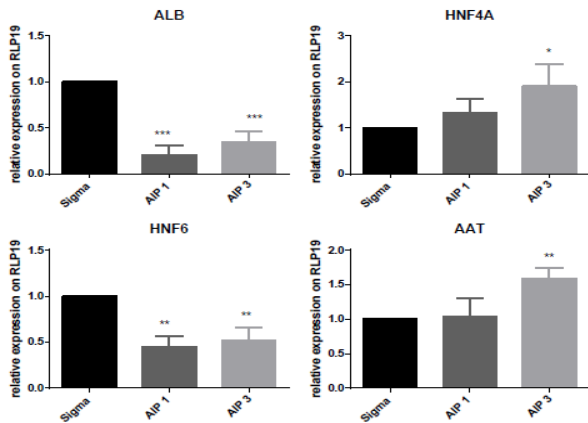
Fig.14 Transcript levels of genes involved in hepatic differentiation. AIP HLCs lines expressed lower transcript levels of *ALB* and *HNF6* and comparable transcript levels for *HNF4* and *AAT* compared to HLCs derived from Sigma control line. The results are reported as mean ± SD of three independent experiments. *RPL19* was used as housekeeping gene. Unpaired t-test was performed after verification of normal distribution with R Shapiro.test. *=pvalue<0,05; **=pvalue<0,005; ***=pvalue<0,001 indicate significant difference between the AIP HLCs and control AIP.

However, the absolute mRNA transcript numbers for *ALB* and *HNF6* were in the general range observed for multiple hepatocyte differentiations in the Verfaillie lab (4000-250/1000 copies of *RPL19* for *ALB* and 250-15/1000 copies of *RPL19* for *HNF6*) for each differentiation experiment.

To further exclude that hepatocyte-like differentiation of AIP lines was defective, we valued the expression levels of genes involved in glycolysis, that are generally more expressed in undifferentiated and dedifferentiation cells. HLCs derived cells from the AIP lines expressed lower transcript levels for *LDHA*, *HKII* and *PFKB3,* related to glycolysis. Moreover, *G6PC* and *PEPCK,* two gluconeogenic genes, were higher expressed in AIP3 HLCs (fig 15).
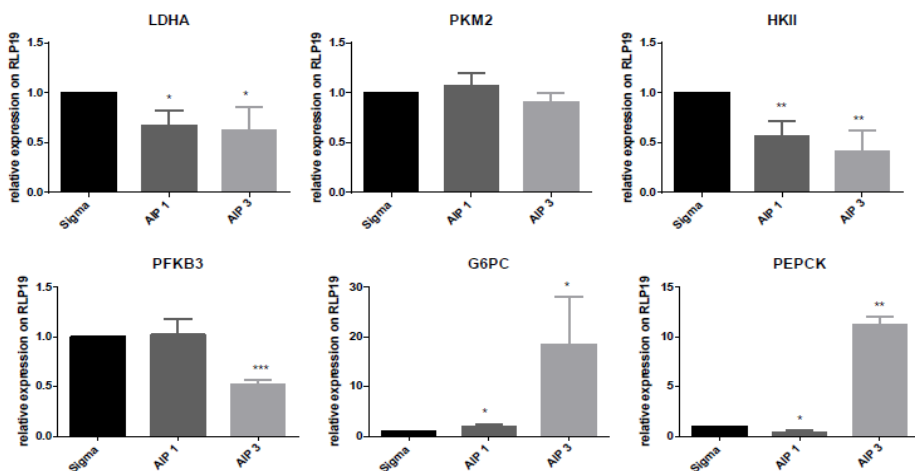
Moreover, we tested albumin secretion and CYP3A4 activity. Although *ALB* transcript levels were lower in AIP HLCs, there was no significant difference in ALB secretion between patient and Sigma control HLCs and CYP3A4 activity was similar as well (fig 16).
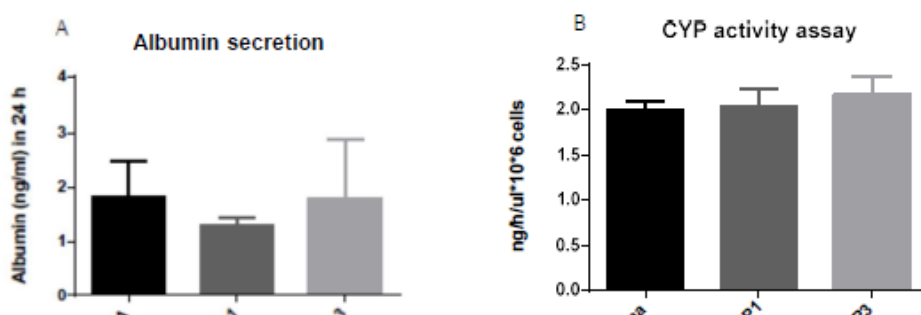

Fig. 16 Functional analysis of AIP1 and AIP3 HLCs derived lines. No difference in albumin secretion and CYP3A4 activity was seen in patient HLC compare to control Sigma HLCs. Results are representative of two independent experiments.

4.2.3 Metabolic pathways in HLCs derived from AIP patients

As acute intermittent porphyria is a disease affecting PBGD, the third enzyme in the heme biosynthetic pathway, we tested the genes involved in this pathway to determine if alterations exist linked with PBGD impairment.

ALAS1 transcript levels were increased in AIP1 and AIP3 HLCs compared to Sigma HLCs. In addition, transcripts for *PPARA* were elevated in AIP3 HLCs, more so than AIP1 HLCs, compared to Sigma HLCs; while expression of *PGC1A, FECH*, *HMOX1* and *TRANSFERRIN* transcripts was elevated only in AIP3 HLCs. In AIP1 was also detected an induction of *HMBS* transcripts (Fig 17).
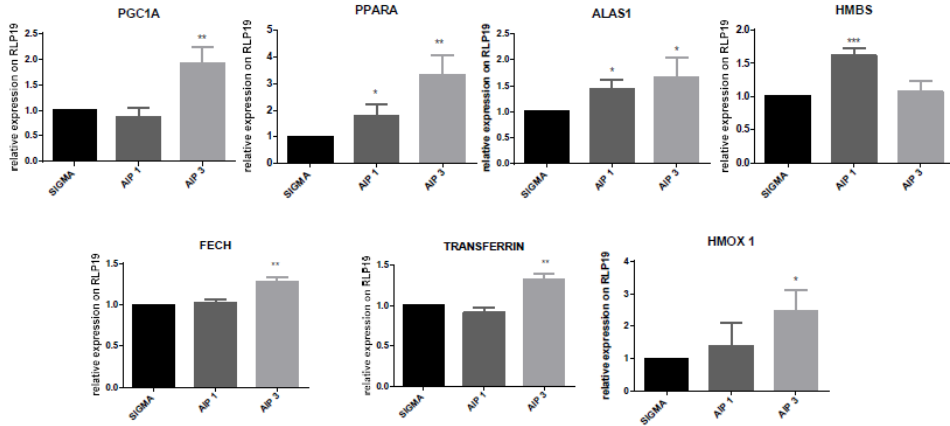
Fig. 17 Heme biosynthetic pathway in HLCs derived cell patient lines. The genes involved in the heme biosynthetic pathway were induced in HLCs derived cells from the patients compared to control Sigma HLCs. Results are reported as mean ±SD of three independent experiments. *RPL19* was used as housekeeping gene. Unpaired t-test was performed after verification of normal distribution with R Shapiro.test. *=pvalue<0,05; **=pvalue<0,005; ***=pvalue <0,001 indicate significant difference between the AIP HLCs and control AIP.

To confirm haploinsufficiency of PBGD in spite of the normal transcript levels for *HMBS*, we tested the activity of the enzyme. The residual activity of the enzyme was lower than 40% in the two patient HLCs derived cells, in line with the diagnosis of acute intermittent porphyria (fig 18).
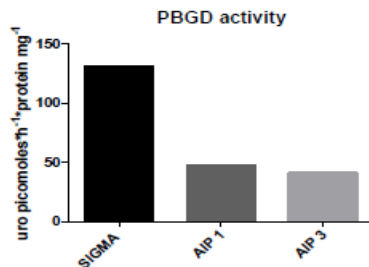

Fig. 18 PBGD activity. The PBGD activity was measured as picomoles of uroporphyrin production normalized on the proteins concentration. N=1.

Interestingly, the AIP HLCs expressed higher levels of *HMGSC*, a gene involved in ketone body formation and cholesterol synthesis. To further investigate these pathways, we analysed the expression of the thiolases, upstream in the *HMGCS* and the transcript level of *HMGCR*, the gene coding for the rate limiting enzyme of the cholesterol biosynthetic pathway.

We found an increased expression of *HMGS2* and *HMGCR* in both AIP HLCs, suggesting an induction of the cholesterol synthetic pathway and fatty acid oxidation. This was substantiated by the observation that also transcripts for *ACAT1* and *ACAT2* in AIP3 HLCs were increased, as well as *ACAA2*, the thiolase specific for ketone body formation (fig 19).
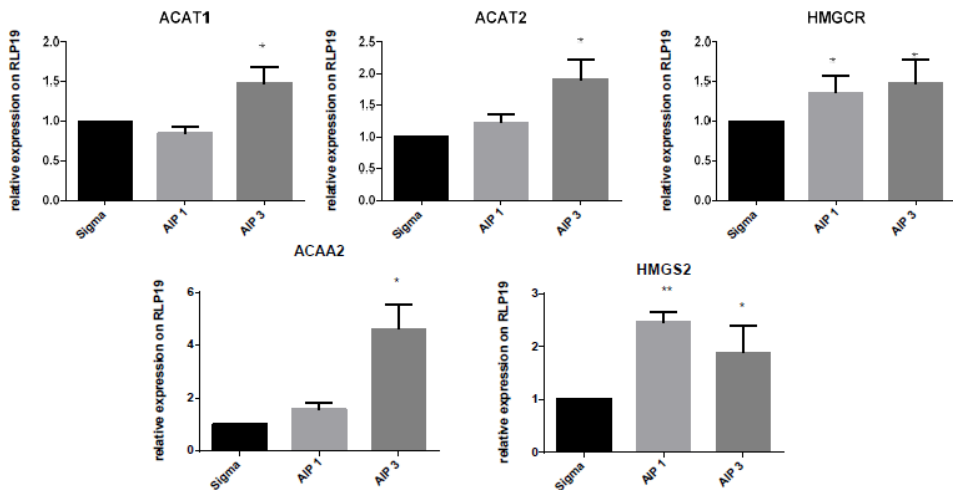

Fig.19 Ketone bodies and cholesterol synthesis related genes. Both HLCs patient lines presented the induction of the *HMGS2*, involved in both ketone body formation and cholesterol synthesis, and the most specific gene for cholesterol synthesis *HMGCR*. The thiolases for these pathways were also induced in AIP3 HLCs. Unpaired t-test was performed after verification of normal distribution with R Shapiro.test. *=pvalue<0,05; **=pvalue<0,005; ***=pvalue<0,001 indicate significant difference between the AIP HLCs and control AIP.

demonstrated elevated levels for *ALAS1* in both patients derived HLCs, as well as transcripts for *FECH* and *HMOX1* in AIP3 HLCs, we tested by qRT-PCR transcript levels for glutathione synthase (*GSS*), glutathione S-transferase (*GST*), uncoupling protein 2 (*UCP2*) and catalase (*CAT*).

Elevated levels of the three genes were found in AIP1 and AIP3 HLCs, suggesting increased oxidative stress possibly related to unstable oxygen-contain molecules (Fig.20)
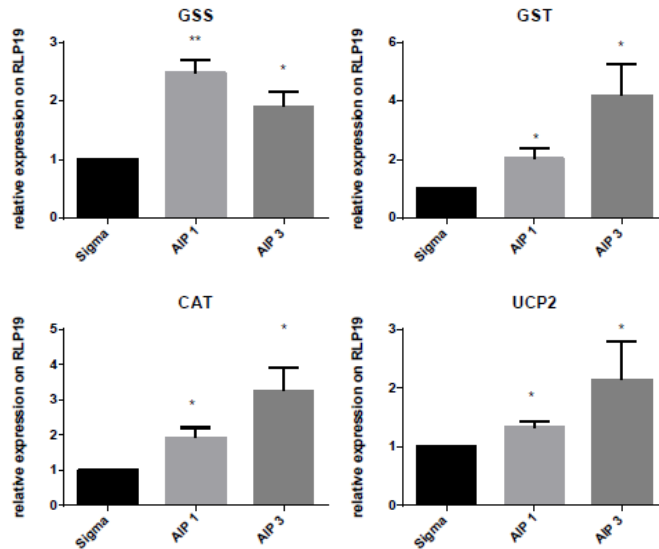

Fig 20 Induction of oxidative stress response in HLCs derived lines from AIP1 and AIP3. Both AIP HLCs expressed higher levels of oxidative damage response genes compare to the HLCs derived from Sigma control line. Unpaired t-test was performed after verification of normal distribution with R Shapiro.test. *=pvalue<0,05; **=pvalue<0,005; ***=pvalue<0,001 indicate significant difference between the AIP HLCs and control AIP.

4.2.4 Effect of 5-delta aminolevulinic acid (ALA) in hiPSCs-derived hepatocytes

To mimic an acute attack and as a result of induction of *ALAS1* in the two HLCs AIP derived lines, we added 300 µM of 5-aminolevulinic acid (ALA) to the to the HLC culture medium for the last 24 hours.

In the HLCs derived from the Sigma control line, we observed an induction of *FECH* and *HMOX-1*, indicating that the ALA provided was used in the heme biosynthetic pathway (fig 21).
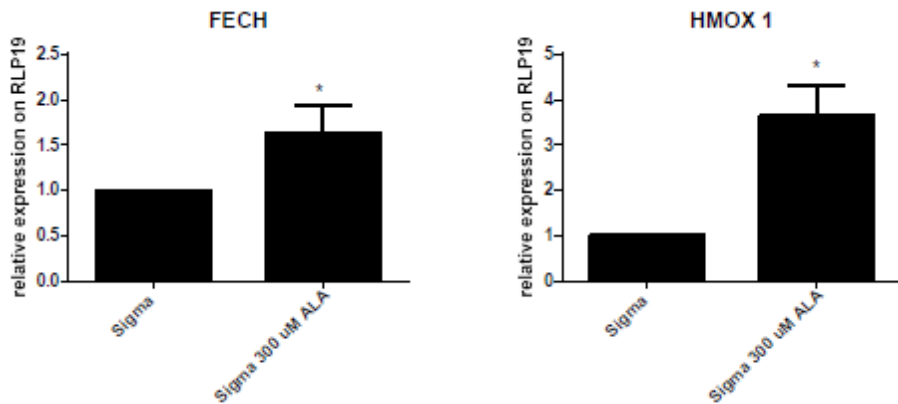
Fig. 21 Induction of heme biosynthesis mediated ALA. In presence of ALA, the heme biosynthetic pathway was induced, as shown by increased levels of *FECH.* In addition, and possibly by increased production of heme, we also observed an induction of *HMOX1*. Unpaired t-test was performed after verification of normal distribution with R shapiro.test. *=pvalue<0,05; difference between Sigma HLCs without and with ALA.

The decreased levels of *ALAS1* may suggest presence of a residual amount of heme acting in the known negative feedback control of *ALAS1.*

The level of *ALAS1* was also consistent with a downregulation of *PGC1A*, which might be caused by increased glucose import due to the increased expression of *GLUT1* (fig 22).
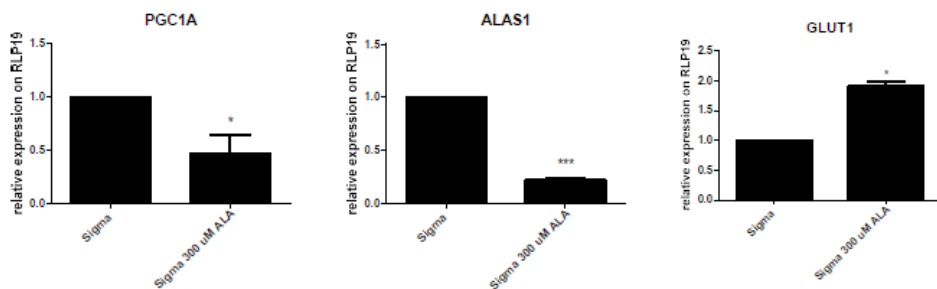

Fig. 22 Effect of ALA on ALAS1. *ALAS1* was downregulated in the presence of ALA as well as *PGC1A*, while *GLUT1* was induced. Unpaired t-test was performed after verification of normal distribution with R Shapiro.test. *=pvalue<0,05; ***=pvalue<0,001 difference between Sigma HLCs without and with ALA.

Moreover, no induction of genes involved in the oxidative stress response pathway was seen, indicating that the oxidative stress observed in the AIP HLCs may not be directly dependent on the presence of ALA.

The gene expression pattern AIP HLCs demonstrated similarities with Sigma HLCs treated with 300 μM of ALA. The induction of heme pathway genes as *FECH* in AIP1 and *BLVR*, the gene involved in heme catabolism, in both treated AIP HLCs were observed. Also in both AIP HLCs, we observed a decrease in *ALAS1 and PPARA* (fig 23) combined with increased levels of *GLUT1* when ALA was added the last 24h (fig 24).
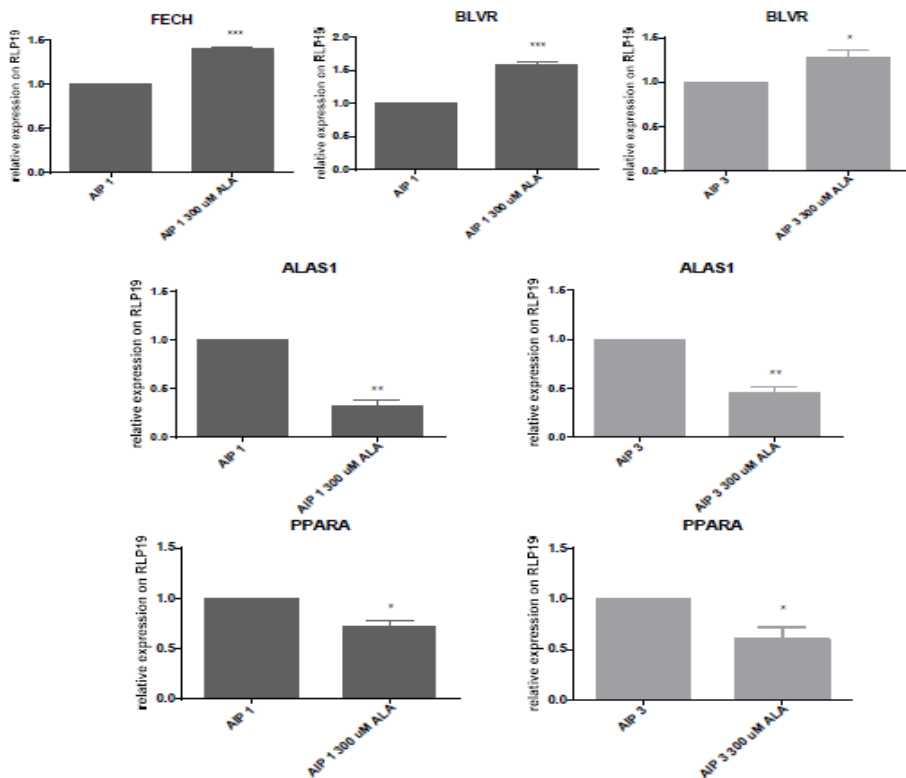

Fig 23 Effect of ALA on heme metabolism related genes. In the presence of ALA there were induction of *FECH* and *BLVR*. *ALAS1* was lower in both AIP-HLCs combined with a down regulation of *PPARA* *=pvalue<0,05; **=pvalue<0,005 ***=pvalue<0,001 indicate significant differences in comparison to the relative AIP-HLCs lines without ALA.
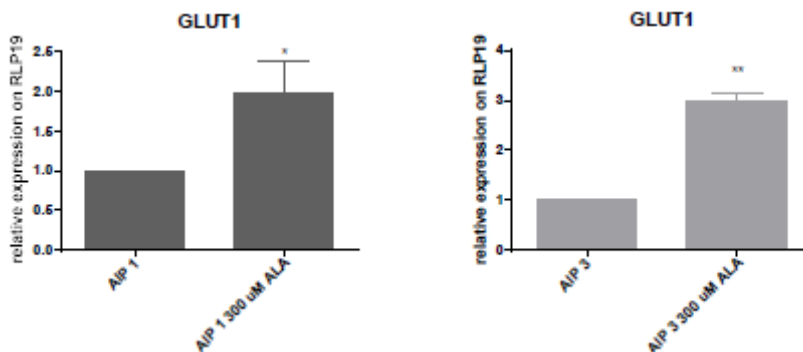
Fig 24 Effect of ALA on GLUT1. In the presence of ALA there was overexpression of glucose transporter *GLUT1* in both AIP-HLCs. *=pvalue<0,05; **=pvalue<0,005 indicate significant differences in comparison to the relative AIP-HLCs lines without ALA.

Finally, in the presence of ALA, we found an induction of the thiolase *ACAT2* (fig 25) in HLCs derived from AIP1 cell lines, and no other variations in the already high level of genes involved in cholesterol and ketone body formation was seen as in normal condition without ALA for HLCs-AIPs (fig 19).
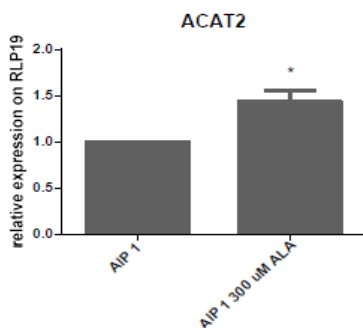

Fig. 25 *ACAT2* overexpression in presence of ALA in the HLC-derived AIP lines. In AIP1 HLCs, an induction of *ACAT2* expression was found following addition of ALA. *=pvalue<0,05 indicates significant differences in comparison to AIP1 HLCs without ALA.

4.2.5 Effect of phenobarbital in HLCs derived cells

One of the inducers of porphyric attacks in acute intermittent porphyria is phenobarbital. Therefore, we added 200 mg/ml phenobarbital the last 24h of the differentiation to mimic the acute attack, and assessed the effects at the transcription of genes linked with the metabolic changes of in AIP HLCs.

Phenobarbital induced gene expression within the heme biosynthetic pathway, including *ALAS1*, *ALAD*, *HMBS* and genes linked with the iron transport *transferrin* (*TF*) and *mitoferrin* in Sigma-derived HLCs. The unchanged expression levels of *HMOX1*, but increased expression of other members of the pathway, suggests that as higher levels of heme are needed, catabolism through *HMOX1* may not be occurring. Induction of *ALAS1* was also associated with overexpression of *PPARA* and *PGC1A* (fig 26).
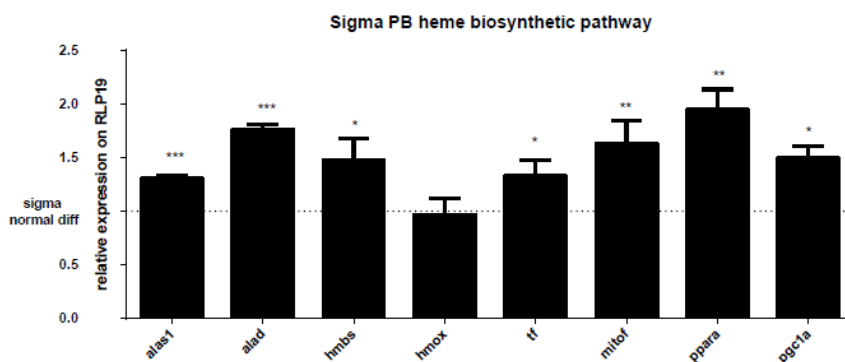
Fig. 26 Expression profile of the heme biosynthetic pathway after phenobarbital (PB) treatment in control Sigma HLCs. PB treatment induced the heme biosynthetic pathway with overexpression of key genes as *ALAS1, ALAD* and *HMBS*, in presence of induction of *transferrin* (*TF*) and *mitoferrin*. Induction of *PPARA* and *PGC1A* was also found following PB treatment. *=pvalue<0,05; **=pvalue<0,005; ***=pvalue<0,001 indicate significant differences in comparison to control Sigma HLCs not treated with PB

PB caused an oxidative stress response in the Sigma HLCs as shown by the increased expression of *GST*, *UCP2*, *ATP5A1*, *CAT* and *GSS* (fig 27).
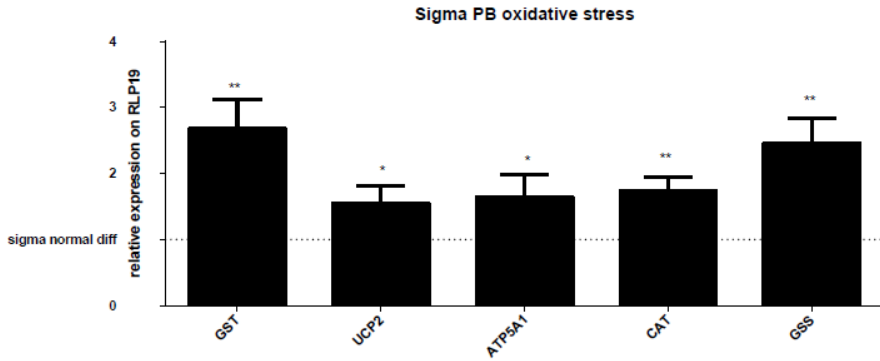
Fig 27 The phenobarbital induce oxidative stress in control Sigma HLCs. After treatment with PB there was induction of genes related with oxidative stress response. *=pvalue<0,05 and **=pvalue<0,005 indicate significant differences in comparison to control Sigma HLCs not treated with PB

More interestingly, after PB treatment the metabolic genes of control Sigma HLCs was changed. We observed an induction of the thiolase *ACAA2* involved in ketone body formation, as well as *ACAT1*, *ACAT2* and *HMGCR* important in cholesterol synthesis. This suggests that these pathways are activated in response of drug metabolism. Moreover, the increased expression of *GLUT2* and *PEPCK* suggest that there may be an increased need for glucose in this metabolic context requiring in enhanced glucose transport and gluconeogenesis (fig 28).

As also seen for the Sigma HLCs, PB induced expression of the *GLUT2* glucose transporter and *PEPCK* in HLCs derived from AIP1 and AIP3 patients iPSCs (fig 29), whereas no further induction of *ALAS1* and its regulator genes were observed after phenobarbital treatment.
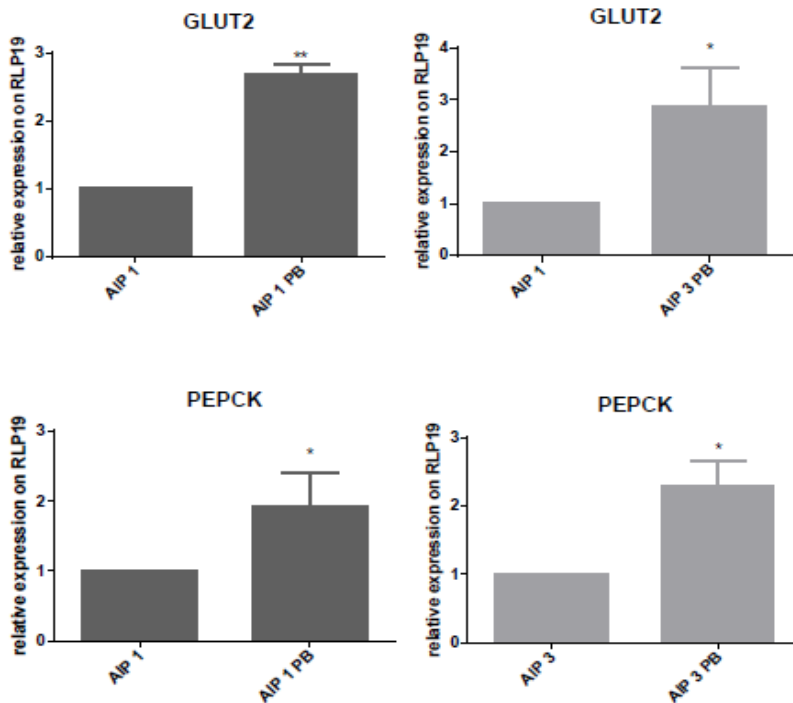
Fig 29 Induction of *GLUT2* and *PEPCK*. The glucose transport *GLUT2* and gluconeogenesis *PEPCK* gene expressions were induced in both AIP-HLCs lines *=pvalue<0,05 and **=pvalue<0,005 indicate significant difference in comparison to relative AIP-HLCs lines without phenobarbital treatment.

Moreover, in AIP3-HLCs, PB also induced overexpression of ketogenic and cholesterol synthesis related genes in presence of induction of *PXR* and one of its target *CYP2C9*, one of the cytochromes involved in phenobarbital metabolism(fig 30).
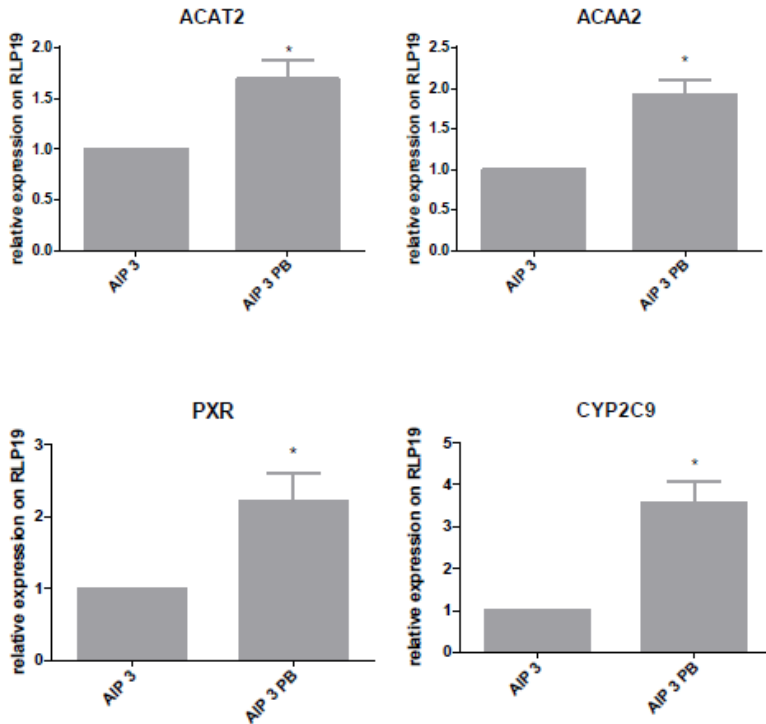
Fig 30 Metabolic changes in HLCs derived from AIP 3 iPSCs. Treatment with PB induced expression of genes involved in ketogenesis and cholesterol synthesis as the thiolase *ACAT2* and *ACAA2*. PB also induces the expression of *PXR* and its target *CYP2C9* involved in drug metabolism.*=pvalue<0,05 indicates significant difference in comparison to AIP 3 HLCs without treatment. N=3

DISCUSSION

Porphyrias are a group of rare disease characterized by a variegate heterogeneity of mutations affecting the genes directly or indirectly involved in the heme biosynthetic pathway[5]. The majority of the mutations described are localised in the coding region of the genes that are included in the routine molecular diagnosis. On the other hands, only few variants in the regulatory regions of the porphyric genes have been reported in association with porphyrias [31][32] despite the high rate of mutation probability due for the less selective pressure on these regions[33]. The effects of variants in the regulatory regions are not predictable as for the coding variants, and required experimental confirmations.

In this study, we proposed a putative role for variants in the regulatory region of PPOX gene identified in five symptomatic patients of variegate porphyria. The data for the c.1-883G>C located in the promoter of PPOX proposed a transcriptional role for this variant. The low expression measured in the patient, correlated with an impairment of the luciferase activity generated by the construct -883C. Moreover, the difference showed between the two different cell line transiently transfected with the construct, supports the different promoter activity in liver and erythroid cells of the PPOX gene previously reported[14]. Even if bioinformatics analysis[34] did not highlighted the loss of transcriptional binding site generated by the presence of the variant, the ChIP-seq database[35] indicate a high-density binding signal for different transcriptional factors. The c.1-883G>C might be involved in the stability of a complex essential for the transcription of the *PPOX* gene. The results for the c.1-413G>C suggest a post-transcriptional role for this variant. In spite of the normal expression of the PPOX in the patient, the transfected construct -413C generated a low activity luciferase signal. This reduction was not concord with the mRNA quantification of the exogenous luciferase vector transfected that was comparable to the wild type construct. The c.1-413G>C variant is located on the proximity of the transcriptional

starting site of *PPOX,* in a region recognized by the pre-initiation complex for the translation of the mRNA[36]. The presence of this variant could modify the binding ability of the complex but further experiments allowing the protein quantification on cells carrying this variant could clarify its effective role.

In conclusion for the first part of the project, we also suggested a splicing modulation action for the c.1-176G>C substitution.

This variant is located in the last nucleotide of the 5'UTR of the variant 2 of the PPOX gene, the most abundant variant isolated in human leucocytes[13]. The sequencing of the isolated mRNA of the patients showed a deletion of 4bp due to the alternative splicing generated by the presence of the substitution. However, no difference in the mRNA expression of *PPOX* in the patients compared to normal controls, confirm a proper maturation of the 4bp deleted mRNA. The localization of the variant and the generated deletion could affect the post-transcriptional regulation of the gene by altering the interaction with cell specific trans-acting protein[37]. The predicted structure [35]of the *PPOX* 5'UTR indicate a stable stem loop structure, that could be targeted by tissue or environmental regulatory proteins. CAGE race experiments in association with minigene transfection assay could confirm the role of the variant in the protein generation.

The second part of my PhD project was focused on the generation of human induced pluripotent stem cells derived hepatocyte like cells (HLCs) to study acute intermittent porphyria.

Human induced pluripotent stem cells (hiPSC) have been proposed as a powerful technology for disease modelling for genetic diseases [22]. The generation of pluripotent stem cells directly from somatic cells of the patients allows the production of tissue-specific derived cells useful for pathophysiology studies *in vitro* using a versatile platform that maintains the genetic background of the patients [38]. Human hepatocyte-like cells derived from hiPSCs were previously used for liver disease in vitro modelling [39], infectious disease studies [40] and drug metabolisation screening[41].

The use of Sendai viral vector-based reprograming constitutes an easy and integration free approach to generate hiPSC, as used in this thesis, from blood samples of two symptomatic acute intermittent porphyria (AIP) patients with recurrent acute attacks.

The hiPSC were differentiated into hepatocytes using a modified differentiation culture condition protocol developed by Ruben Boon (**Engineering of the nutrient microenvironment is indispensable for hepatic maturation of stem cell derived progeny and hepatoma cell lines Under review in Nature Biotechnology)** that improves the metabolic maturation of *in vitro* HLCs, although they maintain some fetal marker expression.

In this thesis, I demonstrated that HLCs showed a comparable expression gene profile with human primary hepatocytes, except for *ALAS1*, the gene encoding for the first rate-limiting enzyme of the pathway that was approximatively 2.5 times lower expressed. This is consistent with the observation by R. Boon, that the metabolic profile of HLCs, despite optimization of the culture conditions remains immature if performed without induction of hepatocyte and metabolic transcription factors. Nevertheless, as harvesting primary liver cells from patients is nearly impossible, and in view of the fact that culture of primary hepatocytes causes a very fast loss of mature hepatocyte functions, in *vitro* HLCs remain a good platform to perform pathophysiology studies applied to hepatic porphyrias.

The only published study performed on hepatocytes from acute intermittent porphyria patients, found a notable overexpression of ALAS1 and HMOX1, without heme depletion [10]. Our results were in line with these data. In HLCs from both AIP patients, *ALAS1* was increased consistently in association with an increase in *PPARA* and *PGC1A* transcripts. *PPARA* and *PGC1A* are known to be involved in *ALAS1* induction [42][43] and are also linked to gluconeogenesis, glycolysis and fatty acid oxidation [44]. Analyzing the gene expression profile of genes involved in glucose metabolism, we noted some

differences compared to the Sigma control HLCs. The AIP HLCs expressed less glycolysis transcripts, and in AIP3 HLCs cells expressed higher levels of *PEPCK*, a gluconeogenesis related gene. Furthermore, in both patient derived HLCs there was an increase in expression of genes involved in cholesterol synthesis and fatty acid oxidation, suggesting a difference in energy homeostasis maintenance also in physiological conditions. These data were supported by previous observations of hypercholesterolemia in AIP patients and altered glucose metabolism. In contrast with data from a mouse model [45], our data suggest that also in basal conditions, and not only after fasting, glucose metabolism in AIP HLCs might be supported by gluconeogenesis and ketogenesis. Therefore, it may be interesting to assess ketobodies accumulation in porphyria patients, in which symptoms might also reflect ketoacidosis due to a crosstalk between the heme biosynthetic pathway and ketogenesis. This hypothesis will obviously require further studies.

The increased expression of genes involved in the oxidative damage response in AIP-HLCs was in line with data observed by Ferrer et al in the circulating patient's cells [46].

To reproduce *in vitro* an acute attack, we used different strategies. We added 300 μM of 5-delta aminolevulinic acid (ALA), described as the concentration produced in hepatocytes during an acute attack [47]. In the ALA condition, the induction of *FECH* and *HMOX1* expression in the control normal HLCs was indicative of increased heme biosynthesis. This was also suggested by the lower expression levels of *ALAS1*. This decrease might be mediated by the negative feedback control of heme on *ALAS1,* the rate-limiting step of the pathway. Interestingly, we found a concurrent induction of the glucose transporter *GLUT1*, which may be indicative of an increased basal glucose requirement upon heme overproduction. The absence of induction of other genes related to oxidative damage in normal control HLCs suggested that other metabolites in porphyria patients must be involved in this process. In

AIP HLCs, the increased need for glucose linked with heme biosynthesis induction in presence of ALA, was also suggested by the increase in gluconeogenesis and ketogenesis gene expression, with, in addition, a constant high level of the transcripts of genes involved in cholesterol synthesis.

We also treated AIP and control HLCs with phenobarbital (PB), one of the porphyrinogenic drugs causing acute attack in acute intermittent porphyria patients. In response to PB, changes seen in control HLCs appeared to reproduce some of the phenotypic alterations seen in the AIP HLCs under non-stressed conditions, including effects on gene expression within the oxidative stress pathway, and induction of ketogenic and gluconeogenic genes, followed also by increased levels of transcripts of genes involved in cholesterol synthesis.

Although induction of cholesterol synthetic genes has been described as consequence of phenobarbital-induced stimulation, the exact mechanism and the reason for this reaction remain unknown. Our observation that *ALAS1* is induced in control HLCs in response to PB and the already base-line increased expression of this gene in the AIP HLCs, suggests the hypothesis that the heme pathway may be involved in the induction of cholesterol synthesis, which could explain the hypercholesterolemia reported in some cases of acute intermittent porphyria [48].

The continuous induction of the heme biosynthetic pathway in HLCs AIP line and the similar metabolic changes observed in the HLCs control line suggested an uninterrupted request of heme in patient lines that was induced in the HLCs control line to support the increased cytochromes synthesis and may be insufficient in AIP lines in basal conditions. We can now, not determine if the impairment of the pathway by a mutation of the *HMBS* gene leads to a heme deficiency. The overexpression of *ALAS1* in the HLCs patient lines also in basal conditions may be due to a block of the negative

feedback mediated by heme, support this hypothesis. The quantification of heme produced will be required to further elucidate this finding.

Homedan et al [9] demonstrated an energetic deficiency in a mouse model of acute intermittent porphyria due to mitochondrial energetic failure. They hypothesised this observation to be a consequence of a cataplerosis of TCA cycle caused by withdrawal of succinyl-CoA by ALAS induction. The oxidative stress observed in AIP HLCs and in control HLCs after PB induction of *ALAS1* supports this hypothesis, not only in an acute attack context but even in basal conditions.

The increased expression of glucose transporters, gluconeogenic and ketogenic genes, reinforce the notion of the important role played of the nutritional status on clinical expression of AIP. Collantes et al [45] described the role of alterations in glucose metabolism in AIP mice: during fasting in the AIP mouse model induced gluconeogenesis and ketogenesis instead of glycogenolysis during nutritional deprivation. In addition, it was assumed that mitochondrial energy failure and the inability of the TCA to supply reduced cofactors might affect the mitochondrial respiratory chain.

Our data support the link between heme biosynthesis, energetic metabolism and nutritional context. However, more experiments are required.

These data support the evidence that porphyrias are genetic complex diseases, in which alterations in other metabolisms contribute to the onset of the disease. The hiPSC derived HLCs to model porphyrias would be an important tool to allow further experiments as RNA-seq analysis applied in an hepatic contest, in which the disease take place. The capacity of generation of different cells type is also important to study the effect of the porphyrins production in different tissues in a genetic patient-specific context. Further, exome sequencing analysis on a large cohort of porphyria patients combined with tissue specific metabolic analysis will be potent devices to characterize the disease in an overall way.

CONCLUSIONS, SHORTCOMINGS OF THE CURRENT STUDY AND FUTURE DIRECTIVES

In this project, we characterized the functional role of three new variants in the regulatory regions of PPOX gene suggesting a transcriptional involvement for the c.1-883 G>C variant and a putative translational effect for the c.1-413G>T. The c.1-176 G>A produce an alternative splicing variant for the PPOX gene that does not affect the mRNA level of PPOX.

These data evidence the inclusion of the regulatory regions in the diagnostic process of porphyrias. Further experiments as electrophoresis mobility shift assay (EMSA) or cell manipulations generated by CRISPR/Cas9 could clarify the direct action of the variants in the transcription and protein level of *PPOX.* The generation of iPSC-derived hepatocytes cells like, are a valid tool to investigate the transcriptional and metabolic alterations involved in acute intermittent porphyria at hepatic level in a patient specific background contest.

However, improvement in hepatic differentiation protocol and further functional and biochemical analysis are required to better understand the metabolic network linked with heme biosynthetic pathway.

The quantification of the glucose consumption and pyruvate and lactate production could better support the crosstalk between heme synthesis, respiratory chain and TCA cycle (experiments ongoing).

Moreover, the measurement of intracellular and released porphyrins (experiments ongoing) could show a link between induction of the pathway and overproduction of porphyrins, responsible of the metabolic changes and probably linked with the symptoms of the disease. To highlight a common response to the different endogenous and exogenous stimuli however, the recruitment of more patients and controls is required.

Another important experiment for the interpretation of the data is the correction of the mutation in the AIP lines to validate the metabolic changes suggested by the current studies. It is well known that the single mutation

does not correlate with the phenotype as evidence of the low penetrance of porphyrias. For that reason, a combined use of the piggy bag transposon vector and CRISPR/Cas9 system to replace the wild type base is proposed as strategy for the correction of the mutations. Further studies on correct lines will have the potential to explain the origin of the metabolic alterations as either connected to the mutation or derived from the genetic background of the patient lines.

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SCIENTIFIC PRODUCTS

Manuscripts

Fiorentino V, Brancaleoni V, Granata F, Graziadei G, Di Pierro E (2016). The assessment of noncoding variant of PPOX gene in variegate porphyria reveals post-transcriptional role of the 5' untranslated exon 1.*Blood Cells Mol Dis.* http://doi: 10.1016/j.bcmd.2016.08.002.

Brancaleoni V, Balwani M, Granata F, Graziadei G, Missineo P, Fiorentino V, Di Pierro E (2015). X-chromosomal inactivation directly influences the phenotypic manifestation of X-linked protoporphyria. *Clinical Genetics.* http://doi.org/10.1111/cge.12562

Oral presentations

Fiorentino V, Brancaleoni V, Granata F, Graziadei G, Cappellini MD, Di Pierro E: "Characterisation of the functional role of variants within the regulatory regions of UROD and CPOX genes".

ICPP September 14th-16th 2015, Dusseldorf, Germany

Poster presentations

Fiorentino V, Brancaleoni V, Granata F, Graziadei G, Cappellini MD, Di Pierro E: "Functional role of unknown noncoding variants in PPOX gene identified in variegate porphyria patients"

ICPP June 25th-28th 2017, Bordeaux, France

Brancaleoni V, Granata F, Fiorentino V, Graziadei G, Di Pierro E: "Molecular Characterisation of Erythropoietic Protoporphyria in Northern Italy".

SIGU October 21th-24th 2015, Rimini, Italy

Brancaleoni V, Granata F, Fiorentino V, Graziadei G, Spinelli D, Fustinoni S, Cappellini MD,

Di Pierro E: "Molecular Characterisation of Erythropoietic Protoporphyria in Northern Italy".

ICPP September 14th-16th 2015, Dusseldorf, Germany

Kurt I, Uyanik M, Brancaleoni V, Fiorentino V, Cappellini MD: "The first case of homozygous

variegate porphyria in Turkey: A novel mutation in the protoporphyrinogen oxidase gene".

ICPP September 14th-16th 2015, Dusseldorf, Germany