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End of Degree Dissertation

# The effects of an LPI environment on *Slc7a7* deficient mice

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**Degree in Biotechnology**

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Location: Barcelona

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# Summary

**Title:** The effects of an LPI environment on *Slc7a7* deficient mice.

**Author:** Katie Jean Wilce

**Supervisor:** Susanna Bodoy

**Date:** June 2021

**Keywords:** LPI, macrophages, $y^+$LAT1, hepcidin, hypoarginemia.

Lysinuric Protein intolerance (LPI) is a rare disorder caused by mutations in the cationic amino acid (CAA) $y^+$LAT1 transporter gene. LPI has a high range of complications which include a malabsorption and reabsorption of CAAs and a urea cycle defect that leads to low levels of arginine and high ammonium in plasma. Many immune system dysfunctions are phenotypes of this disease and are still far from understood. It was seen in previous studies that alveolar macrophages in LPI accumulate iron and that the iron regulator hormone hepcidin is expressed more in LPI mice than in control mice. This dissertation studies the mechanisms of regulation that could be allowing these high concentrations of plasma hepcidin while also analysing macrophages in an LPI environment and their activation pathways. We used a tamoxifen inducible knock out mouse (CreUBC) as well as a myeloid cell line knock out mouse model (LysM). Liver hepcidin expression and some of its regulatory genes were analysed in these 2 models. The expression of Hamp1 (hepcidin gene) and its regulatory genes showed no differences in the LysM model, indicating that it should be the LPI environment that are the route of the problem and not the lack of the transporter in the macrophages. There was a tendency in a higher expression of hepcidin and some regulatory genes in the total knock out animal but no statistical difference, so the experiment needs to be repeated for a sure conclusion. The macrophages in LPI environment of arginine showed no statistical differences between genotypes in macrophage activation but the results are not completely viable due to manipulation errors and must be repeated to reach a reliable conclusion.

# Resumen

**Título:** Los efectos de un entorno LPI sobre ratones deficientes en *Slc7a7*.

**Autora:** Katie Jean Wilce

**Tutor:** Susanna Bodoy

**Fecha:** Junio de 2021

**Palabras clave:** LPI, macrófagos, $y^+$LAT1, hepcidina, hypoarginemia.

La lisinuria con intolerancia a proteinas (LPI) es una enfermedad rara causada por mutaciones en el gen SLC7A7 que codifica para un transportador de aminoácidos catiónicos $y^+$LAT1. LPI tiene una gran variedad de complicaciones, pero las alteraciones básicas son una malabsorción y reabsorción de aminoácidos catiónicos y un defecto del ciclo de la urea que da lugar a baja concentración de arginina y alta en amonio en plasma. Muchas alteraciones del sistema inmunológico son caracteristicas de esta enfermedad y aún están lejos de comprenderse. En estudios previos se observó que los macrófagos alveolares en LPI acumulan hierro y que la hormona reguladora del hierro hepcidina se expresa más en ratones LPI que en ratones de control. Este trabajo fin de grado estudió los mecanismos de regulación que podrían estar permitiendo estas altas concentraciones de hepcidina en plasma, al mismo tiempo que analiza los macrófagos en un ambiente LPI y sus vías de activación. Usamos un ratón knock-out inducible por tamoxifeno (CreUBC), así como un modelo de ratón knock out de línea celular mieloide (LysM). La expresión de hepcidina y algunos de sus genes reguladores se analizaron en ambos modelos. No se observaron diferencias en la expresión del gen que codificapara la hepcidina y sus genes reguladores en el modelo LysM, lo que indica que puede ser el entorno LPI el que afecta a los ratones y no la falta del transportador en los macrófagos. Se vio una tendencia a una mayor expresión del gen de la hepcidina y algunos genes reguladores en el animal knock-out total, pero sin diferencias estadísticas, por lo que el experimento debe repetirse para una conclusión segura. Los macrófagos en el ambiente LPI no mostraron diferencias estadísticas entre genotipos en la activación de macrófagos, pero los resultados son preliminares y se tendrían que repetir para obtener una conclusión fiable.

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## Abreviations

NO: Nitric Oxide

SLC: Solute Carrier

LPI: Lysinuric Protein Intolerance

4F2hc: Heavy chain of superficial antigen 4F2

gpaAT: Glycoprotein Associated Amino acid transporter

$y^+$LAT1: System $y^+$ L Amino acid Transporter.

CAT: Cationic Amino acid Transporter

HAT: Heteromeric Amino acid Transporter

rBAT: Related to $b^{o,+}$ Amino acid Transporter

CAA: Cationic Amino Acid

PAP: Pulmonary alveolar proteinosis

HLH: Hemophagocytic Lymphohistiocytosis

MAS: Macrophage Activation Syndrome

WAT: White adipose tissue

CreUBC: Cre-recombinase ubiquitin C promotor knock out model

LysM: Cre-recombinase Lysozyme Myeloid knock out model

LPS: Lipopolysaccharide

FPN-1: Ferroportin-1

qPCR: Quantitative Polymerase Chain Reaction

BMDM: Bone marrow derived macrophages

M-csf: Macrophage colony stimulating factor

LPS: Lipopolysaccharide

UTR: Untranslated region

IRE: Iron responsive element

RPM: Red pulp macrophages

RNA: Ribonucleic Acid

DNA: Deoxyribonucleic Acid

cDNA: complementary Deoxyribonucleic Acid

PBS: Phosphate Buffered Saline

DMEM: Dulbecco's Modified Eagle Medium

FBS: Foetal Bovine Serum

DTT: Dithiothreitol reducing agent

# 1. Introduction

## 1.1 Aminoacids and their transporters

Amino acids are essential for the human body. They serve not only as building blocks for proteins but as regulators of metabolic pathways and nucleic acid synthesis (Wu, 2009). Arginine in particular plays a crucial role as a signalling molecule inside the cell as it's a precursor for both l-ornithine and nitric oxide (NO) synthesis and a key regulator of the mTORC1 pathway. The transport of amino acids in kidney and intestine is critical for the supply of amino acids to all tissues and the homeostasis of plasma amino acid levels (Bröer, 2008).

Cellular arginine availability is determined by members of the solute carrier (SLC) 7 (Jungnickel et al., 2018). SLC7 family members can be divided into two major groups: cationic amino acid transporters (CATs) and glycoprotein-associated amino acid transporters (gpaATs), or also called the light chain of heteromeric amino acid transporters (HAT's) (Figure 1). The associated heavy subunits (glycoproteins) 4F2hc or rBAT of HATs form the SLC3 family.

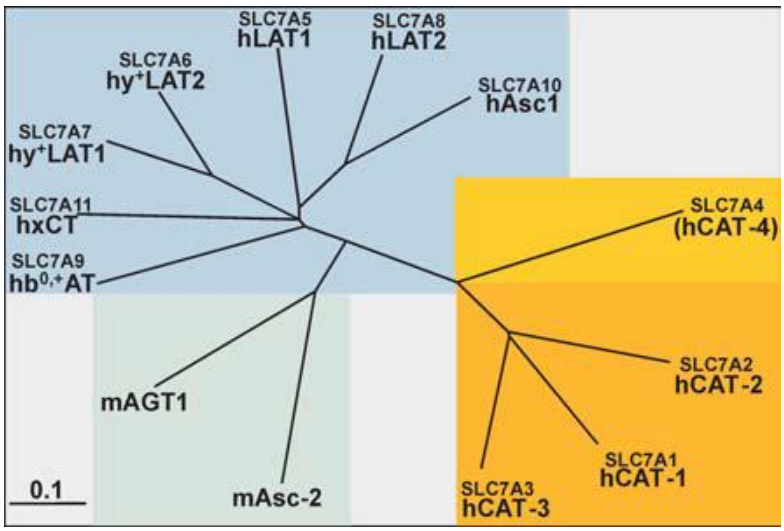


*Figure 1:Phylogenetic tree of the SLC7 family proteins. Figure taken from* (Verrey et al., 2004)

Unlike the CAT's that can function as monomers in the plasma membrane, the gpaATs are obligate heterodimers. This is because the glycoprotein functions to traffic the transporter to the plasma membrane and aid in protein stability (Fotiadis et al., 2013).

Members of the CAT family transport mainly cationic amino acids by facilitated diffusion with differential stimulation by intracellular substrates. In contrast, heterodimeric amino acid transporters have a large selectivity in terms of substrate (Table

1). They can range from large neutral amino acids (system L), to small neutral amino acids (system asc), negatively charged ones (system $xc^-$) and cationic plus neutral amino acids (system y+L and $b^{0,+}$ like). Mutations in $b^{0,+}$ and $y^+L$ transporters lead to hereditary diseases like cystinuria and lysinuric protein intolerance (LPI) (Verrey et al., 2004; Wagner et al., 2001).

| Transport System | Light Subunit | Heavy Subunit | Tissue/Localization | Function | Pathophysiology | Reference No. |
|---|---|---|---|---|---|---|
| L | LAT1 (*SLC7A5*) | 4F2hc (*SLC3A2*) | Kidney, spleen, thymus, liver, small intestine, placenta, testis, brain, heart, lung, blood-brain barrier, leukocytes/basolateral | $Na^+$-independent exchange of large neutral aa, transport of thyroid hormones; BCH inhibitable | | 9, 66, 68, 90, 112, 121 |
| L | LAT2 (*SLC7A8*) | 4F2hc (*SLC3A2*) | Kidney, placenta, ovary, small intestine, brain, liver, spleen, prostate, testis, skeletal muscle, heart, lung/ basolateral | $Na^+$-independent exchange of smaller neutral aa; BCH inhibitable | | 4, 108, 116, 121 |
| $y^+L$ | $y^+$LAT1 (*SLC7A7*) | 4F2hc (*SLC3A2*) | Kidney, small intestine, leukocytes, lung, erythrocytes, placenta/basolateral | $Na^+$ + large neutral aa/ dibasic aa exchange | Lysinuric protein intolerance ($y^+$LAT1) | 11, 104, 142 |
| $y^+L$ | $y^+$LAT2 (*SLC7A6*) | 4F2hc (*SLC3A2*) | Brain (glia, neurons), small intestine, testis, parotis, heart, kidney, lung, liver/ basolateral | $Na^+$ + neutral aa/dibasic aa exchange; glutamine/ arginine exchange | | 16, 142 |
| $x_c^-$ | xCT (*SLC7A11*) | 4F2hc (*SLC3A2*) | Macrophages, liver, kidney, brain, retinal pigment cells/ basolateral | Glutamate/cystine exchange | | 3, 12, 126 |
| asc | ascAT1 (*SLC7A10*) | 4F2hc (*SLC3A2*) | Brain, lung, placenta, small intestine, kidney/basolateral | $Na^+$-independent exchange of small neutral aa, also D-serine, D-glycine | | 52, 91 |
| $b^{0,+}$ | $b^{0,+}$AT (*SLC7A9*) | rbAT (*SLC3A1*) | Kidney, small intestine, brain/ apical | $Na^+$-independent exchange of neutral/dibasic aa; cystine, arginine, lysine, and ornithine reabsorption | Cystinuria type I (rbAT) and non-type I ($b^{0,+}$AT) | 25, 47, 103, 117 |

All transport systems are composed of 2 subunits, a light chain and a heavy chain. The name in parentheses gives the Human Genome Organization nomenclature for the respective gene. BCH, 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid; aa, amino acid.

*Table 1: Amino acid transport systems expressed by members of the heteromeric amino acid transporter family. Table taken from* (Wagner et al., 2001).

The gene *Slc7a7* encodes for the $y^+$LAT1 (GenBank RefSeq: NP_003973) cationic amino acid transporter (Mykkänen et al., 2003). When heterodimerized with 4F2hc (GenBank RefSeq: NP_002394), the $y^+$LAT1 protein allows the exchange of cationic amino acids (arginine, lysine, and ornithine) with neutral amino acids plus sodium, typically in the epithelial cells of kidneys and intestines (Figure 2). It's an obligatory co-transport. It has a molecular weight when dimerized of 135kDa. Of note, the transporter is also expressed in macrophages where it also mediates the transport of arginine (Barilli et al., 2010). So, this gene is mainly expressed in the basolateral membrane of epithelial cells from kidney and intestine, and also in macrophages (Wagner et al., 2001).

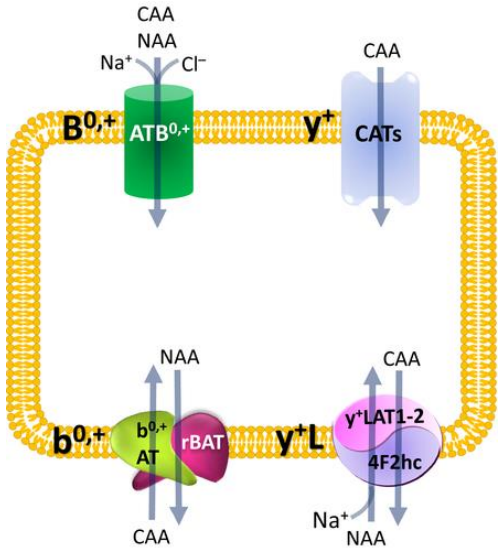


*Figure 2: Schematic representation of transport systems for cationic amino acids in a hypothetical cell. Figure taken from* (Rotoli et al., 2020).

## 1.2 Lysinuric Protein Intolerance

Lysinuric Protein Intolerance (LPI: OMIM 222700) is a rare disease characterized by a mutation in the *Slc7a7* gene that encodes for the light subunit of this cationic amino acid (CAA) transporter (Torrents et al., 1999). Patients with this disease show symptoms such as vomiting, aversion to protein, episodes of diarrhea and of stupor and coma after a protein-rich meal, poor feeding, aversion to protein-rich food, failure to thrive, hepatosplenomegaly, and muscular hypotonia (Nunes & Niinikoski, 1993).

This disease has an especially high prevalence in Finland where the incidence rate is 1/60.000 births and also in southern Italy and the northern part of Japan (Ogier de Baulny et al., 2012; Sebastio et al., 2011).

Patients with this mutation show classical hallmarks by having a leakage of CAAs in urine and normal to low plasma levels (Simell, 2001). This can be ascribable to the malabsorption and hyperexcretion of these amino acids due to the impaired absorption and reabsorption of CAA in the intestine and kidney respectively (Tanner et al., 2007). As arginine and ornithine are part of the urea cycle, shortage in these lead to a deficiency of the urea cycle which can explain the hyperammonemia that it causes (Palacín et al., 2004).

It used to be considered a rather benign urea cycle disease when treated with a low protein diet and citrulline supplementation, because citrulline can be used as an

intermediate for the urea cycle (Simell, 2001). But during recent years, other manifestations show that it is a multiorgan disorder with a variety of clinical symptoms (Bröer, 2007). Another interesting point is that there doesn't appear to be any genotype-phenotype relations, because patients with the same mutations show a wide range of symptoms, for example Finnish patients, all with the same homozygous mutation have a high heterogeneous phenotype (Tringham et al., 2012). Due to this variable and non-specific phenotype LPI is often under- and misdiagnosed (Ogier de Baulny et al., 2012).

Classic clinical symptoms of LPI in infants are neurodigestive signs usually after weaning. They are usually symptom free while breast feeding due to the low protein concentration in human milk (Tanner et al., 2007). Patients show an evident aversion for protein rich food. Due to this nutritionally poor diet, the result is malnourished infants or children with pallor, poor muscle tone, osteopenia and subsequently osteoporosis (Parto et al., 1993). Hepatosplenomegaly typically develops within the first months of life.

Two major complications involve the life threatening complication of pulmonary alveolar proteinosis (PAP), which is due to an impairment of alveolar macrophages as they fail to clear surfactant in alveolar spaces (Barilli et al., 2010) and renal disease. Other complications of LPI include Hemophagocytic lymphohistiocytosis (HLH) and Macrophage activation syndrome (MAS). HLH is primary if it is inherited and secondary if it is related to other diseases, which would be the case of LPI. HLH can progress to MAS if it is not carefully monitored (Lerkvaleekul & Vilaiyuk, 2018). Immune dysfunction potentially attributable to NO overproduction secondary to arginine intracellular trapping (due to defective efflux from the cell) might be a crucial pathophysiological route explaining many of LPI complications (Ogier de Baulny et al., 2012).

Aside from that, alveolar macrophages have also been found to accumulate iron in LPI patients (Parto et al., 1994).

Due to the lack of understanding of many of the LPI alterations in macrophages, my lab found the need to generate a viable animal model.

### 1.3 Animal model

The only previous mouse *Slc7a7* deficient model available as a constitutive knock-out of the gene lead to neonatal lethality (Sperandeo et al., 2007) and was not

viable. The lethality of the murine model was shown to be due to intrauterine growth restriction. The only two mice that survived showed growth retardation and a metabolic derangement identical to that found in LPI which had a fast onset and rapidly caused the death of both animals when exposed to a normal diet instead of a low protein one with citrulline supplementation. Due to this, my lab team found a need to produce a viable model for in vivo studying of LPI.

A self-generated tamoxifen inducible LPI mouse model that recapitulates the main hallmarks was fully available in the lab. More importantly, the mouse model also reproduces the side effects of this disease.

The mouse model we used is fed for 7 days with a tamoxifen diet, because the tamoxifen induces the *Cre* protein that will eliminate the floxed-*Slc7a7* gene. After that, the animal is fed with a low protein diet (8% casein) for 10 days with or without citrulline (Bodoy et al., 2019). They are fed with citrulline in drinking water because citrulline is an intermediate for the urea cycle that can be converted into arginine or ornithine, and it is correctly absorbed because it is a neutral amino acid.

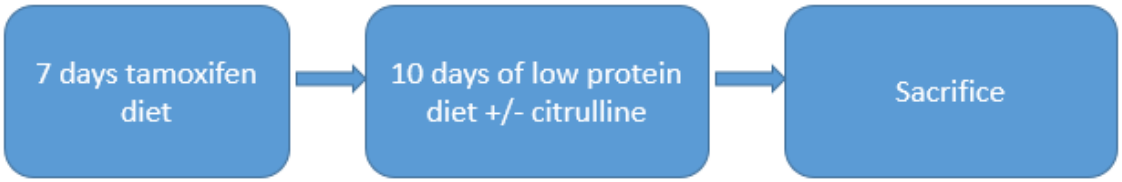


*Figure 3: Animal model treatment before sacrifice.*

The mice were fed a low protein diet to avoid complications. *Slc7a7*$^{-/-}$ mice with a low protein diet showed a reduced survival after the tamoxifen induction. This was due to a dramatic decrease in body and white adipose tissue (WAT). When these mice were supplemented with citrulline, the survival was 100%, as it improved the metabolic derangement present in the previous mouse model (Bodoy et al., 2019). This allowed the survival of the *Slc7a7* induced mice until they were then later sacrificed. Interestingly, citrulline supplementation is also the first line of treatment for LPI patients (Lukkarinen et al., 2003).

Another animal model we used is the Lysozyme model (LysM) in which *Slc7a7* is depleted only in the myeloid cell line, allowing us to see the effects of $y^+$LAT1 depletion in macrophages only, with the remaining expression in kidney and intestine.

## 1.4 Macrophages

Macrophages derive from blood monocytes that leave the circulation to differentiate in different tissues (Geissmann et al., 2010). They are involved in detecting, phagocyting and destroying bacteria and other harmful organisms and they are also antigen presenting cells that can initiate inflammation by presenting antigens to T-cells. They also release cytokines that activate other cells. They are specialized cells within each population of macrophage depending on the tissue (Table 2).

| Type of macrophage | Location | Function |
|---|---|---|
| Alveolar macrophage | Lung alveoli | Phagocytosis of small particles, dead cells or bacteria. Initiation and control of immunity to respiratory pathogens |
| Kupffer cells | Liver | Initiate immune responses and hepatic tissue remodelling. |
| Microglia | Central nervous system | Elimination of old or dead neurons and control of immunity in the brain. |
| Splenic macrophages (marginal zone, metallophilic and red pulp macrophages) | Spleen marginal zone, red and white pulp | Elimination of dysfunctional or old red blood cells. |

*Table 2: Macrophage types and functions in different tissues. Table taken from the British society of immunology.*

### 1.4.1 Macrophage activation

The polarity of macrophages controls the expression of either the classical or the alternative pathway.

The classical pathway, also known as M1 macrophages, are known for being responsible for inflammation response. They respond to proinflammatory stimuli like LPS for example, which is a sugar from a pathogen (Meng & Lowell, 1997).

The alternative pathway, which are the M2 macrophages, are anti-inflammatory and play a role in oxidative phosphorylation. The polarity is induced from monocytes to M2 thanks to IL4 or IL-13, cytokines that are produced in a Th-2 type response (Varin & Gordon, 2009).

Each of these trigger the expression of different transcription factors which is useful for us to determine which type of macrophage has emerged in different situations (Klei et al., 2017; Platanitis & Decker, 2018). M1 express genes such as *TNF-α*, *IL-6*,

*IL1*-β, *NOS2* and *COX2*, because these are pro-inflammatory cytokines. So, when there is a pro-inflammatory stimulus, the macrophage releases these cytokines. M2 activated macrophages express *TGF-β, Arg1*, *Mrc1* and *Ym1* amongst others. These genes are anti-inflammatory and promote growth such as the mannose receptor (Mrc1) and the TGF-β. These repurpose arginine metabolism to express ornithine and polyamine (Figure 4) (Loke et al., 2002).

Macrophages are one of the other cells that express $y^+$LAT1, meaning that they need CAAs to function, especially arginine. The metabolism of arginine in macrophages determines a lot about the immune system pathway that could happen. LPI patients suffer from many immune system disorders, and it would be interesting to investigate more on the function of macrophages in LPI.

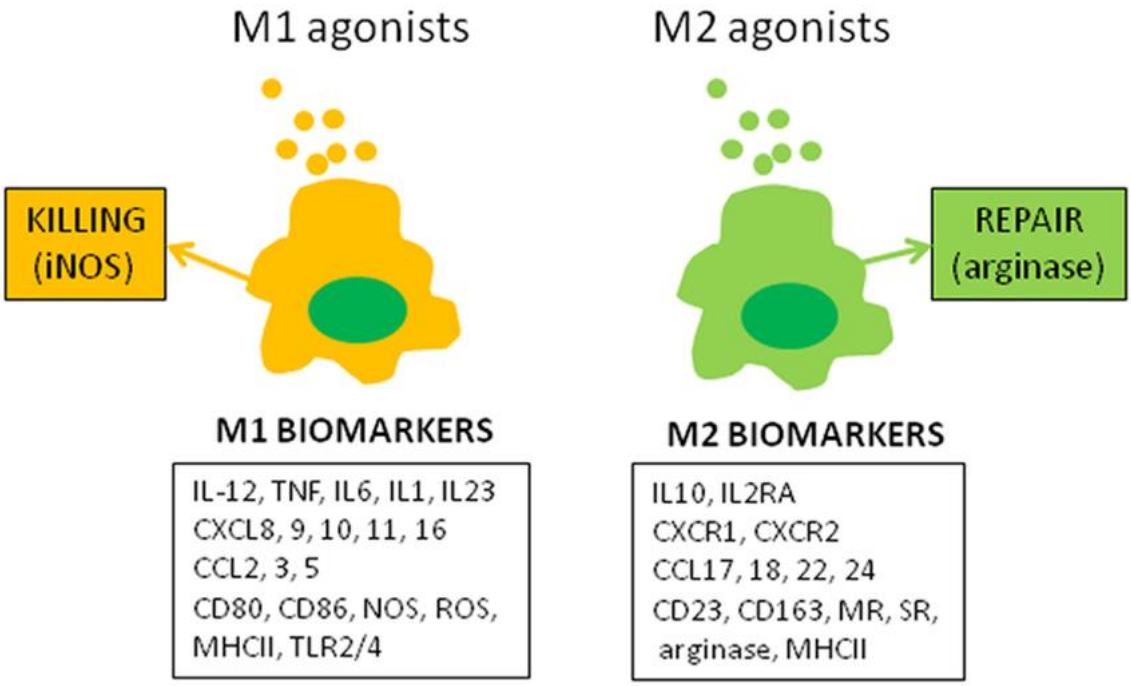


*Figure 4: Polarization of monocyte to M1 or M2 macrophages. Figure taken from* (Ka et al., 2014).

### 1.4.2 Arginine metabolism in macrophages

Arginine is one of the most versatile amino acids in our organism due to its many metabolic fates (Figure 5). Arginine serves as a precursor for synthesis of protein, nitric oxide, creatine, polyamines, agmatine, and urea (Morris, 2006). These processes aren't expressed in each cell and are differentially expressed depending on each cell type. Arginine metabolism is modulated thanks to transporters (like $y^+$LAT1) that move it across the plasma membrane with its metabolites.

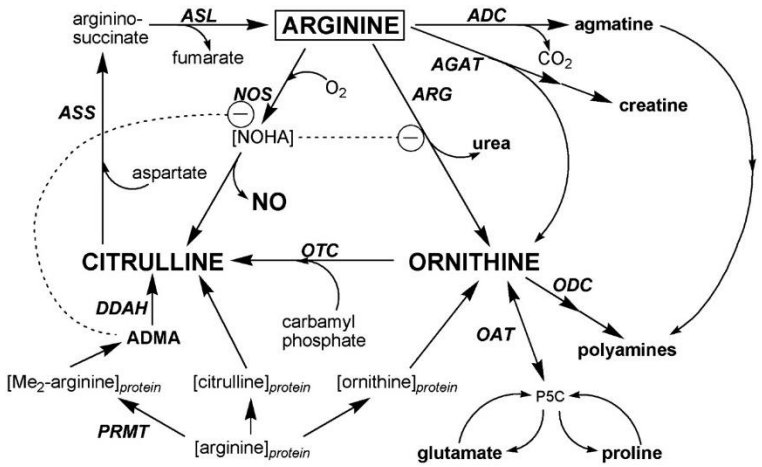


*Figure 5: Overview of mammalian arginine metabolism. Figure taken from* (Morris, 2007).

Macrophages produce nitric oxide which is then used to kill pathogens. That NO (nitric oxide) comes from arginine as you can see in figure 5. Thanks to other stimulations, arginine in macrophages can give way to ornithine, and in the liver can give way to urea for the urea cycle. Ornithine can stimulate cell proliferation (via polyamines) and aid in repair processes (via collagen) (Mills, 2001).

Although the primary role of NO is to protect against harmful pathogens, it can also interfere with the immune system responses by inhibiting lymphocyte replication. In some cases it also appears to induce apoptosis (Brüne, 2003).

As mentioned before, the other major pathway of arginine catabolism in macrophages through arginase, results in the production of ornithine. Ornithine has been shown to promote tumour cell replication and lymphocyte proliferation (Bowlin et al., 1987; Pegg, 1988).

As we have seen the importance of arginine in macrophage function, we can begin to understand why a low arginine concentration environment such as LPI metabolic condition can have an effect on the immune system of LPI patients.

### 1.4.3 Regulation of iron metabolism in macrophages

Aside from the before mentioned importance of arginine metabolism in macrophages, another important thing to note is that in the LPI mouse model, alveolar macrophages and red pulp macrophages have been observed to accumulate iron. This could be due to the high rate of erythrophagocytosis by red pulp macrophages (unpublished results from the lab). Most of the iron that enters plasma daily comes from

macrophage-recycled iron. The only iron exporter known in macrophages is Ferroportin-1 (FPN-1) (Ward & Kaplan, 2012).

FPN-1 is the only known non-heme iron exporters that releases iron into the blood stream in mammals. It's expressed in almost all body cells, as most cells require a way to export excess iron but it's expressed at particularly high levels in cells that must export large quantities of iron as part of their normal daily activities, including enterocytes, macrophages of the reticuloendothelial system, and hepatocytes (Collins et al., 2018).

Ferroportin is regulated at many different levels. At post translational level it's regulated by the hormone hepcidin (De Domenico et al., 2011) through the systemic iron status, whereas intracellular iron availability regulates ferroportin synthesis via the 5′ untranslated region (UTR) iron responsive element (IRE) of the ferroportin mRNA (Hentze et al., 2010). Hepcidin is a hormone predominantly synthesized by the liver, which oversees the degradation of FPN-1 (Ogun & Adeyinka, 2018). This means that if there is a high expression of hepcidin, then there will be a state of anaemia, because it is degrading FPN-1 which is not allowing iron to be released into the blood. And on the contrary if there is a low expression of hepcidin then we often reach high levels of iron in blood (Hentze et al., 2010).

Hepcidin is basically regulated by the transcription of its gene (*HAMP1*) (Sangkhae & Nemeth, 2017). It is regulated in a few known ways: by Inflammation, by erythropoietic activity and by iron (Figure 6). Inflammatory cytokines are ones such as IL-6 which regulate the transcription through the STAT pathway. BMP6 from the iron signalling ultimately regulates the expression of hepcidin (Andriopoulos et al., 2009) through the joining to HJV triggering the SMAD pathway that induces the transcription of hepcidin. Also, we have IRE which binds to iron and activates hepcidin transcription.

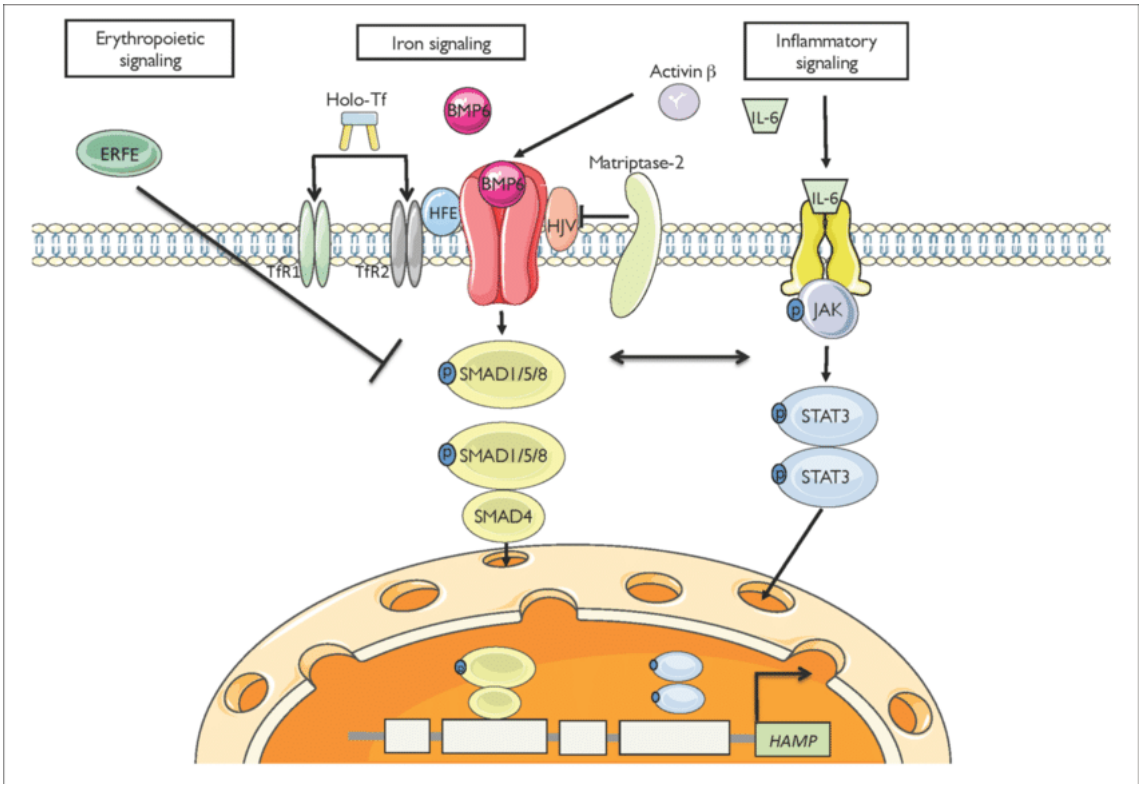


*Figure 6: Major pathways of hepcidin regulation. Figure taken from* (Sebastiani et al., 2016)

# 2. Objectives

## 2.1 Background

As we have seen in the immune complications of human LPI, previous results show that there is an iron accumulation in macrophages, and the expression of the iron regulating hormone hepcidin in plasma is high in *Slc7a7*$^{-/-}$ mice (Figure 7). Because of this, we are interested in knowing why hepcidin is high and what mechanisms are regulating this. Previous results from the group also show that inflammatory cytokines IL-6, which is known to regulate hepcidin expression, is equal in plasma between genotypes.

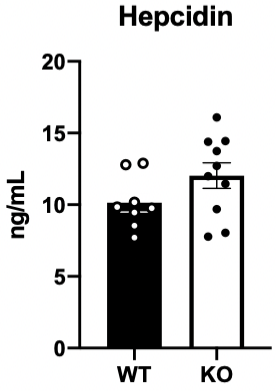


*Figure 7: Hepcidin concentration in plasma (ng/mL) in wild type mice (black bars) and knock out mice (white bars) from previous results of the lab. Data represents mean +/- SEM. Graph made using GraphPad Prism.*

We are also aware of the macrophage dysfunction in the LPI mouse, being the exacerbated erythrophagocytosis in red pulp macrophages (RPM) and the surfactant accumulation in alveolar macrophages causing PAP. This is why it interests us to know more about macrophage function in LPI, as it's clearly a main cause for problem. Many studies have been done on a myeloid line (LysM) knock out model and it seems to show that the mouse functions the same, meaning it's not the *Slc7a7* deficient macrophages themselves causing complications. It's the LPI environment affecting the function of macrophages which as mentioned previously is known for being high in ammonium due to the hyperammonemia caused from urea cycle deficiency and low in arginine concentrations because of the hypoarginemia from the malabsorption and reabsorption in intestines and kidneys.

## 2.2 Objectives

The different objectives of my study are:

- Validate genotypes of our animal model.
- Analyze the expression of regulatory genes of Hepcidin (*HAMP1)* in liver in *Slc7a7*$^{-/-}$ and *Slc7a7*$^{LysM-/-.}$
- Characterize Bone Marrow Derived Macrophages (BMDM) activation in LPI environment.

# 3. Methodology

## 3.1 Design of the experiment

The design of the experiment consisted firstly of generating a mouse model which recapitulated the main LPI hallmarks in humans (Bodoy et al., 2019). To induce the Cre recombinase to knock out the expression of *Slc7a7*, the knockout mice were fed a tamoxifen diet for 7 days and then a low protein diet for around 10 days, and finally they were sacrificed.

From there we collected the organs we needed such as the spleens, as this is the organ that is the main productive machinery of red pulp blood cells, the bone marrow, which allowed us to study macrophage function as we used the bone marrow to differentiate cells to macrophages, the kidneys seeing as it's one of the tissues that expresses the $y^+$LAT1 protein when it's not knocked out and the liver because hepatocytes are the main players in hepcidin synthesis.

In each experiment we had to assure the genotype of the animal to know if it was a successful knockout or a wild type animal. We assess that by always performing a western blot against the $y^+$LAT1 protein before being able to carry on with the results from our experiments. The antibody is handmade from the lab.

## 3.2 Western blot

One of the first steps of realizing our studies is first knowing my animal model well. We must perfect a series of western blots to check if my animals are correctly knocked out or if they are wild type. We do this by using the kidneys because it is one of the tissues which most expresses $y^+$LAT1 and we perform a membrane protein extraction, because if we performed a total protein extraction our protocol would not be successful due to the fact that our antibody is handmade, so the sample needs to be pure as it isn't good enough for total protein extraction. $y^+$LAT1 is a transporter present on the membrane.

### 3.2.1 Membrane protein extraction

Membrane protein extraction was performed on frozen tissues and the homogenization step was performed using a membrane buffer (25mM HEPES, 250mM sucrose and 4mM of EDTA with Protease Inhibitor Cocktail Set III, Animal Free Calbiochem at a 1:1000 dilution). Samples were homogenized in the lysis buffer with 3

beads to lysate the tissue, then centrifuged for 10 minutes at 10000rpm at 4ºC to eliminate cell debris and finally the supernatant was centrifuged using an ultracentrifuge and a TLA55 rotor for 1 hour at 4ºC and 55000rpm. The pellet was resuspended in 100µL of lysis buffer using a 25G syringe.

See appendix for full protocol.

### 3.2.2 Protein quantification

Once extracted we can quantify and evaluate our proteins. The samples were quantified using the Pierce BCA Protein Assay kit (Thermo Scientific, Ref: 23225, Waltham, MA, USA). The principal of the BCA assay is based on the biuret reaction. Proteins have the capacity to reduce Cu2+ to Cu1+ in an alkaline solution and results in a purple solution. The concentration of protein is then read thanks to a spectrophotometer which reads the absorbance at 450nm.

See appendix for full protocol.

### 3.2.3 SDS-PAGE electrophoresis

SDS-Page electrophoresis stands for sodium dodecyl sulphate–polyacrylamide gel electrophoresis and it's a method which uses the combination of SDS and acrylamide to cancel out the influence of structure and charge so that proteins are separated solely using their mass. In this way, proteins with a lower mass travel at a faster pace through the gel and the heavier proteins stay towards the top of the gel. Our membrane proteins were resolved in a 10% acrylamide gel for the SDS page. We loaded 30µg of protein per well.

See appendix for full protocol.

### 3.2.4 Membrane transfer and immunodetection

The transfer of the western blot is what gives it the name. The proteins from the electrophoresis gel are transferred to a blotting membrane which will then carry all of the protein bands originally on the gel. This allows us to work with the proteins using immunodetection. The membrane we use are the Immobilion-E (Catalogue No: IEVH85R) which does not need to be previously activated with methanol.

The membranes are blocked using 5% milk in PBS for an hour. This prevents any nonspecific reactions from occurring because the milk contains a high amount of proteins. The membranes must be handled only using tweezers and not be touched by our hands.

Once the blocking is complete we use a polyclonal rabbit antibody against mouse $y^+$LAT1 proteins, which was handmade in the lab, and the β-actin primary antibody that we used as a control (Sigma) which we probe overnight at 4ºC with our membranes at a dilution of 1:1000 for the $y^+$LAT1 antibody and 1:10000 for the β-actin antibody.

After washing membranes 3 times with PBS for 10 minutes we incubate with the secondary antibodies (anti-rabbit for $y^+$LAT and anti-mouse β-actin) for 1 hour and wash again 3 times for 10 minutes with PBS. We have to make sure that the membrane is always wet so that it doesn't dry out.

Proteins were detected using Amersham ECL Western Blotting Reagents and Amersham Hyperfilm ECL films.

See appendix for full protocol.

#### 3.2.5 Membrane stripping

If we want to re-do the immunodetection because the revelation didn't appear to work as we wanted, we can do a process of striping. This is simply the process in which we strip the membrane of the antibodies we used to detect so we can start again with the membrane only containing the proteins from the gel.

To strip we wash the membrane for 10 minutes in PBS and then wash 3 times with boiling water. We can then proceed back to the step of immunodetection in which first we block for 1 hour with milk and then leave with the corresponding antibodies.

### 3.3 Quantitative PCR

We also want to analyse the expression of different genes in WT control mice and LPI model mice. To do so we will perform a reverse transcription quantitative PCR in combination with Real time PCR. We use extracted RNA from spleens, macrophages, liver or kidneys to see the expression of genes.

In macrophages we will concentrate especially on genes that are expressed depending on the polarization towards M1 macrophages or M2 macrophages. M1 express

genes such as TNF-α, IL6, IL1-β, NOS2 and COX2, and M2 express TGF-β, Arg1, Mrc1 and Ym1. These are the genes we checked the expression of in macrophages.

In the liver we analysed the expression of hepcidin regulating genes such as *Hamp1*, BMP6, IL-6 and IL1-β. In other tissues we evaluated the expression of *Fpn-1*, $y^+$LAT1, CAT2 (another cationic amino acid transporter) … Although not all results were used in my analysis. Hepcidin is only expressed in liver, so we do not check for hepcidin expression in these other tissues.

### 3.3.1 RNA extraction

We must extract the RNA from our samples because this will allow us to see all the messenger RNA present in that sample, allowing us to see what genes have been transcribed and so are active in our tissue. When working with RNA it's important to keep a clean workspace and use RNAse free materials.

The Trizol reagent kit was used for the homogenization steps on tissues. Trizol is a very toxic substance, so its manipulation was always done under a biological safety cabinet and all materials used while manipulating it were thrown away in a specific toxic waste bin. We must first homogenize the sample which involves a complete disruption of the tissue. This step stops degradation of RNA. Once it's homogenized, we must perform the lysis, this step allows the breakdown of the cell to release all of its components. You get left with proteins, DNA and RNA which needs to be purified to obtain only RNA.

With cell cultures, for the lysis of the cells a lysis buffer supplemented with 1% DTT (a reducing agent that breaks disulphide bonds) was used (Invitrogen, Ref: 46-6001) and each plate scraped vigorously, then each sample passed through a 25G syringe.

After the lysis of the samples, the RNA purification was performed using the PureLink™ RNA mini kit (Ref: 12183025). The advantage of using this kit as opposed to other kits is that dangerous reagents such as phenols are not necessary.

See appendix for full protocol.

RNA is very sensitive, so once the RNA is obtained if it is going to be manipulated, it must be kept on ice. If it needs storing it must be stored at -20ºC or -80ºC if it is for longer periods of time.

### 3.3.2 Quantification and assessment of RNA

After obtaining of RNA, it must be quantified, and its integrity must be determined. This was done using a Nanodrop ND-100 with only 2µL of sample. The Nanodrop uses photometry based on the intrinsic absorptivity properties of nucleic acids (in our case RNA). Nucleic acids absorb light with a characteristic peak at 260nm. It then uses Beer-Lambert's equation to calculate the concentration of nucleic acid in samples. It also provides us with purity ratios-A260/280 and A260/230. These measure the presence of protein contamination or carbohydrates and phenols respectively. A ratio of around 2 is considered “pure”.

The quantification allows us to then calculate the volume of RNA to perform the retro-transcription into cDNA. We used a quantity of 2 µg of RNA for the liver samples and 0.5 µg of RNA for the cells due to the low number of cells we have in culture versus in tissue.

### 3.3.3 Retro-transcription

The step of retro-transcription is done because to do a Real-Time PCR which is our end step in qPCR, it’s hard to find specific RNA polymerases that transcribe RNA, so we use this step to retro-transcribe our RNA to cDNA. The specific enzyme that does this reaction is the retrotranscriptase, which joined with oligonucleotides and primers can use our RNA as a mould to synthesize cDNA. In this experiment we used the QuantaBio qScript cDNA supermix which contains a blend of random primers and oligo-(dT) primers. The oligo-(dT) primers recognize the polyA tail of messenger RNA and the addition of random primers assures a higher probability of transcribing the whole sequence, due to the read limit that the enzyme tends to have.

The final volume per strip tube is 20µL, always containing 4µL of Supermix and then calculated volumes of water and RNA. We use the thermocycler to retrotranscribe our RNA to cDNA.

See appendix for thermocycler settings.

### 3.3.4 Real time PCR

In this step, the cDNA produced previously is put through a separate step of real-time quantitative PCR. This is a PCR which amplifies our sample according to a specific gene as we provide the primers for the specific gene we want to amplify, and the number

of cycles it takes to reach the threshold is calculated. The less cycles, the more quantity we had initially of cDNA, meaning there was more quantity of RNA in our original sample. The quantification is always relative to a housekeeping gene.

We used MicroAmp Optical 384 well plates (Ref: 4309849) to load our samples with our primer mixes using the SYBR Green PCR Master Mix (Thermofisher) and analyzed in a QuantStudio 6 Flex Real-Time PCR system (Thermofisher Scientific). 10ng of cDNA was loaded per well so once the cDNA was transcribed we had to make sure to do the correct dilution in water depending on the starting amount of RNA we had. β-actin was used as an internal control and in some cases cyclophillin was used for extra security. Quantification cycle values were determined and $2^{-\Delta Cq}$ values were calculated.

## 3.4 Cell cultures

When working with cell cultures, the whole environment must be sterile to avoid any type of contamination that can interfere with the growth of our cells. To do so, we use a biological safety cabinet for all these processes. The material used inside the cabinet is autoclaved, the surfaces and material entered the cabinet are wiped downed and sprayed with 70% ethanol and UV light is used for 10´prior to any usage of the cabinet. Any biological residues are also treated with 20% bleach.

In our case, we are using bone marrow from our animal model to grow macrophages so that we can see the effects certain inductions can have on macrophage polarization in *Slc7a7* deficient mice compared to WT mice without other hormones such as hepcidin playing a factor.

All mediums used in cell cultures must be heated to 37ºC prior to use. The DMEM complete medium used is supplemented with 10% FBS (Foetal bovine serum) to give extra nutrients to the cells and 1% Penicillin/ Streptomycin to avoid any bacterial contaminations or growths.

### 3.4.1 Generation of bone marrow derived macrophages.

Once the mouse has been sacrificed, the bones must be peeled and they extract the femur and tibia, as these are what we will use to extract bone marrow. They make sure to cut a hole in each of these bones and put them in 0.5mL epi with wholes cut in the bottom

with a needle which in turn will be put in 1.5mL epi. They make sure to put 2 bones per epi.

We centrifuge these so that the bone marrow is flushed to the bottom of the 1.5mL epi so now we have pure bone marrow to differentiate to macrophages.

The bone marrow is then resuspended and filtered and we perform erythrolysis on the cells to destroy and dilute the red blood cells, allowing for a purer sample, with no extra stimuli like phagocytosis. To make sure the lysis works well, the buffer must be resuspended very well and the pellet must then be white, if there is any red colour the lysis hasn't functioned correctly.

The cells are plated after washing with PBS and left for 6 days to differentiate into macrophages. To produce the L-cell used in the medium that contains the M-csf (Macrophage colony stimulating factor) we can either use a recombinant one or one that we culture ourselves. We cultured our own in our lab.

See appendix for full protocol.

### 3.4.2 Obtaining of L-cell for bone marrow differentiation.

To obtain macrophages we must use the bone marrow collected from our mice and differentiate the cells to macrophages. The first step is to produce the macrophage colony stimulating factor (M-csf), we do this using the L929 cell line. We must defrost these at room temperature and add them to a 15mL falcon with complete DMEM, which we mix ourselves. The cells are centrifuged, and we keep the pellet which contains all of our cells. After resuspending them we plate them in a 15cm plate with 15mL of medium.

The compound that these cells produce will allow us to differentiate our bone marrow cells to macrophages. But before we can collect the supernatant (L-cell) which contains the M-csf, we must awaken the cells by doing 2 passes, this means passing the cells to duplicate them, and by the third one they will be ready to produce M-csf. To do that we simply take out the medium, rinse once with PBS and add some more medium to the plate. Scrape the plate until all cells should be risen, then replate in different dilutions and different plates. I did this on day 3 and then day 5. They can't have a higher confluence than 80% and we must use a medium of 40mL to plate them to then collect the M-csf.

Once the cells had been passed 3 times and have the right confluence (Figure 8), I plated around 60 plates, all containing 500.000 cells per plate in 40mL of DMEM complete medium.

After leaving them another 7 days, I proceeded to collect the supernatant, which would now be the final product we call L-cell containing M-csf. I put the supernatant in a 2L sterilized bottle. Due to the high amount of supernatant we had, instead of centrifuging as per the protocol suggests (Trouplin et al., 2013) we put it through a 500mL filter unit of 0.2µm which allowed us to filter any dead cells that could be present in the supernatant. We then stored the L-cell in 50mL falcons to be frozen before use.

The L-cell that I created using this protocol will be later tested on some mice bone marrow to see if the differentiation to macrophages is successful.

See appendix for full protocol.

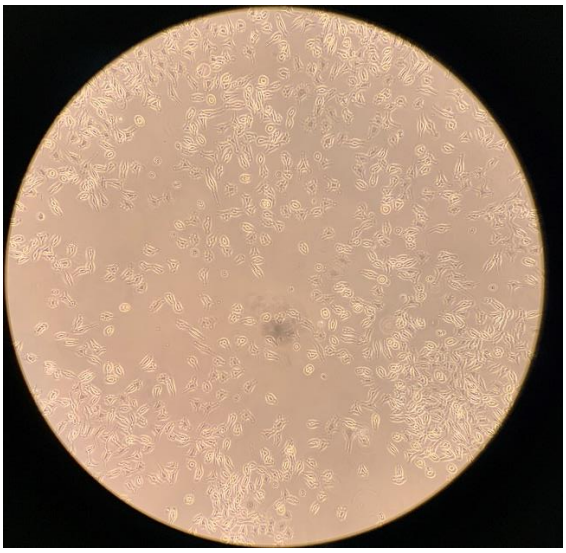

*Figure 8: L929 cells after 3rd pass before seeding. Magnification X10.*

### 3.4.3 Induction of BMDM with LPS or IL4

We are interested in seeing if macrophages without *Slc7a7* expression polarize differently in case they don't have the correct response to an infection or a tissue repair. For this, we incubate the macrophages with M1 and M2 inducers (LPS and IL4 respectively) and see the liberation of different genes that should respond to them. We also want to plate them in different concentrations of arginine to mimic an LPI environment which is low in arginine and have controls.

After the BMDM have been untouched for 6 days, we can go on to plating them for the induction.

Following the protocol in the appendix, we plated 2 million cells per well in complete DMEM and using the Invitrogen countless to count the cells. Cells must be left for 2 hours to seed properly to the plate.

Once the 2 hours are up we swap the complete medium to the corresponding medium per plate. For each animal a medium of 400µM of arginine as a control of excess arginine, 50µM which would be the concentration of a normal non LPI mouse and 20µM of arginine which mimics the low concentrations of arginine in an LPI mouse. Aside from that, for each concentration of arginine, we have 3 different wells which will be used one for a basal, which contains no induction as a control, another one for the LPS induction, activating the M1 pathway and finally another well for the IL-4 induction which activates the M2 pathway.

The inductions were done at a 1:1000 dilution and we put 2mL of medium in each well. I did one timing, so after 1 hour was up, I proceeded to perform RNA extraction to begin the quantitative PCR analysis.

See appendix for full protocols.

### 3.5 Statistical tools

Results from qPCR were calculated using excel, the relative quantities to our housekeeping gene were corrected and then analysed using GraphPad Prism version 9.0.1.

Quantification of band intensity from western blot was analysed using ImageJ by relativizing all samples to one after calculating the percentage of the peaks of each band.

## 4. Results

### 4.1 Validation of $y^+$LAT1 deletion in LPI model

The obtaining of tissues from the mouse model was done through a series of surgical techniques which allowed the extraction of tissues such as the spleen, the femur and tibia for bone marrow differentiation, the kidneys, and the liver. Before proceeding with any experiment, it was important to validate the genotype of the animal to assure it matches up with what we expect and the results we are looking for. The animal model is an induced

knock out through tamoxifen, which doesn't have an efficiency of 100%. For this reason, once we've extracted the kidneys, we proceed to extract the membrane proteins, which is where our protein of interest mainly resides and do the western blot technique as mentioned previously on each animal that interests us to know whether it's *Slc7a7* deficient or not. We used a specific antibody against the $y^+$LAT1 protein encoded by *Slc7a7* that was created by people in the lab and is not commercial.

The samples for the western blot were prepared without using a reducing agent such as DTT, so our protein of interest here appears coupled with its heavy chain 4F2, meaning the molecular weight of our protein of interest is at 140kDa.

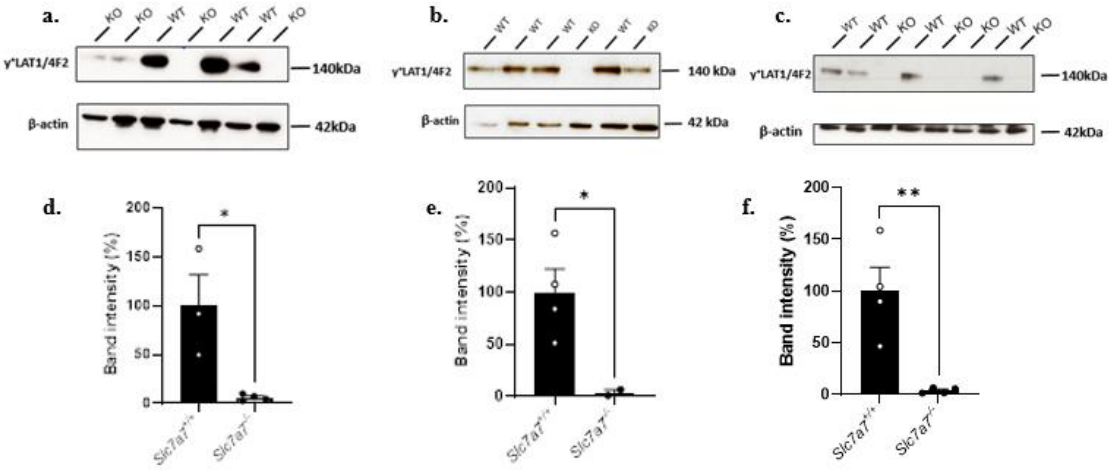


*Figure 9: Western blot revelation with respective percentage of depletion. A, B and C: Film after revelation against y+LAT1 protein of kidney membrane protein extraction using β-actin as a control. D, E and F: Intensity of western blot bands of respect*

In figure 9a, 9b and 9c, the lack of the band at the level of 140kDa shows us the complete depletion of the expression of $y^+$LAT1 heterodimerized with 4F2hc.

In the last lane of the western blot film of figure 9b, you can see a band in the 140kDa level, and the expected genotype of this mouse was a knockout mouse. The actin levels are also not completely the same throughout all the lanes, so this last lane is considered knock out because relative to the quantity of actin that it contains it does have a depletion.

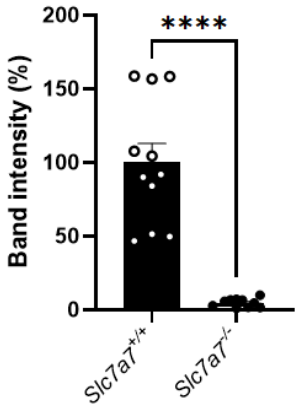


*Figure 10: Band intensity in percentage as a total of previous animals analysed to validate the consistency of $y^+$LAT1 depletion analysed by Western Blot. (****p<0.0001)*

Figure 10 confirms the consistency of the depletions by doing the mean of all of the wild type mice I analysed and all of the knockout ones. Clear statistical difference in the depletion of $y^+$LAT1 was observed, and the model was fully validated.

Once the western blots were done, we can now proceed with the analysis.

## 4.2 Analysis of Hepcidin regulation in liver

As mentioned previously, the levels of hepcidin in plasma of the LPI mice were found to be higher than in the normal mouse model. This discovery stems from the fact that alveolar macrophages and red pulp macrophages were found to accumulate iron in LPI patients and the only known iron transporter in mammals is Ferroportin-1 (FPN1). Amongst other levels of regulation, the post-translational regulation of FPN1 is through the hormone hepcidin. Because these levels were found to be high in LPI mice, it interested us to see what pathway could be regulating this high expression. In both the LPI mouse model and the LysM mouse model we analysed the expression of *Hamp1* and some of its key inducers in the liver. We analysed BMP6 as it regulates *Hamp1* through the SMAD pathway, IL-6 regulates it through the STAT pathway and IL1-β induces hepcidin transcription as well.

### 4.2.1 LysM mouse model

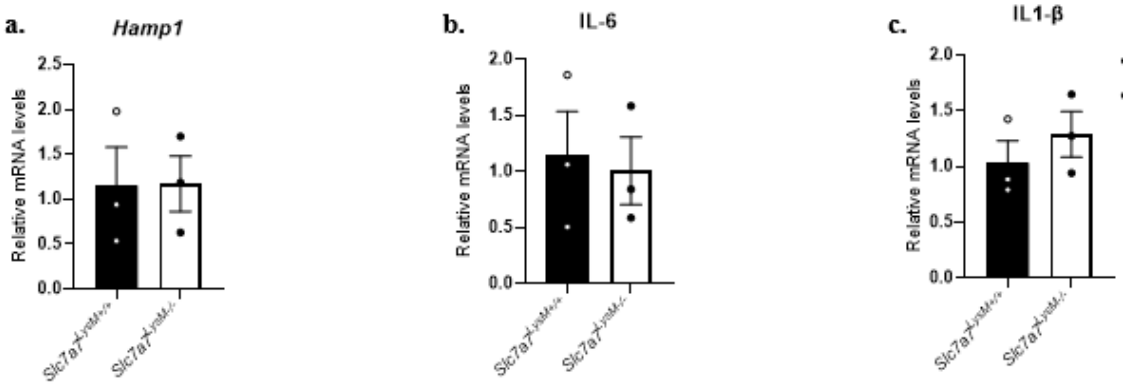


*Figure 11: Relative mRNA levels of Hamp1 and regulatory genes in LysM model using RNA from liver. RNA extraction was performed on liver followed by retro transcription and real time PCR using β-actin and cyclophilin as housekeeping genes to do relative quantification. Values are mean +/- SEM. Graphs made using GraphPad Prism. A: mRNA levels of Hamp1, B: mRNA levels of IL-6, C: mRNA levels of IL1-β.*

The LysM model only has a tissue specific knock out of the gene *Slc7a7*, in macrophages, having the expression normal in kidney and intestine. This can allow us to understand better whether it's the LPI environment affecting macrophage dysfunction or the actual lack of the y+LAT1 transporter in macrophages. The results are from total RNA extraction in liver using 3 control animals and 3 knock out animals. BMP6 results were not used in the LysM model due to possible lack of primer during manipulation in the qPCR stages.

In figure 11a you can see that there are no statistical differences in the expression of *Hamp1* or and in 11b and 11c there is clearly no difference in the expression of *Hamp1* regulatory genes between genotypes.

### 4.2.2 CreUBC mouse model

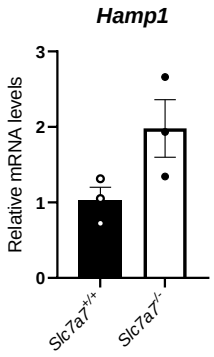


*Figure 12: Relative mRNA levels of Hamp1 gene in wild type mouse (black bars) against Slc7a7 knock out mouse (white bars). RNA extracted from liver. β-actin was used as a housekeeping gene for relative quantification. n=3 WT, 3 KO (p=0,0851). Data represents mean +/- SEM. Graphs made using GraphPad Prism.*

No statistical differences between the genotypes were observed but there is a clear tendency that the knock-out mice show a higher expression of *Hamp1*, with a pvalue close to being statistically different. (Figure 12). A significant difference was expected due to the high levels of the protein found in *Hamp1* protein (hepcidin) in plasma (data obtained previously in the lab).

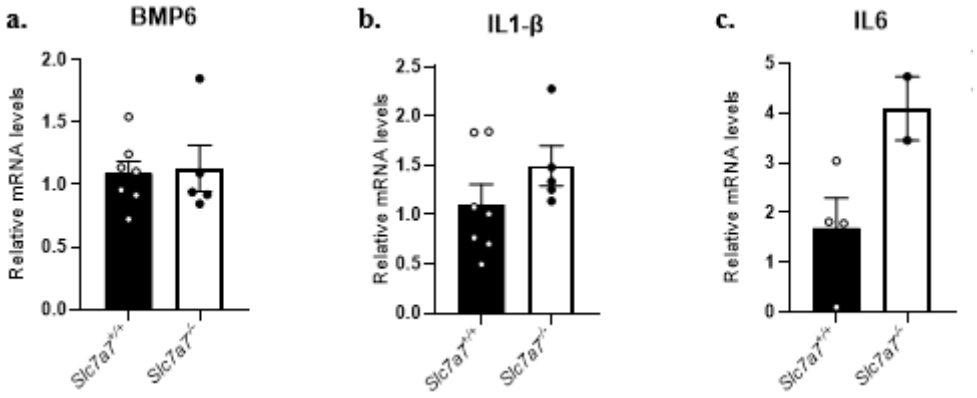


*Figure 13: Relative mRNA levels of Hamp1 regulatory genes against housekeeping genes b-actin and cyclophilin for relative quantification via qPCR. RNA extraction was performed on liver samples and specific primers were used. A: mRNA levels of BMP6, B: mRNA levels of IL1-β, C: mRNA levels of IL6. Data represents mean +/- SEM. Graphs made using GraphPad Prism.*

Figure 13 shows no statistical differences in the expression of these hepcidin regulatory elements with the only one close being IL6 that has a pvalue of 0,0713 which is close to being statistical (Figure 13c). But this cannot be confirmed as in this case there are only 2 knock out animals against 4 wild type animals. This would need to be repeated with a higher number of samples before being able to reach any conclusions.

### 4.3 Characterizing BMDM activation in an LPI environment.

Aside from the western blot to analyse the expression or not of the $y^{+}$LAT1 protein in tissues, simultaneously the BMDM from the LysM model (in which there is only depletion in macrophages) must be checked. As previously mentioned, the differentiation of bone marrow to macrophages is possible thanks to the use of M-csf from the L-cell of cell line L-929 in which we leave bone marrow cells to grow in with complete DMEM for 6 to 7 days (Figure 14).

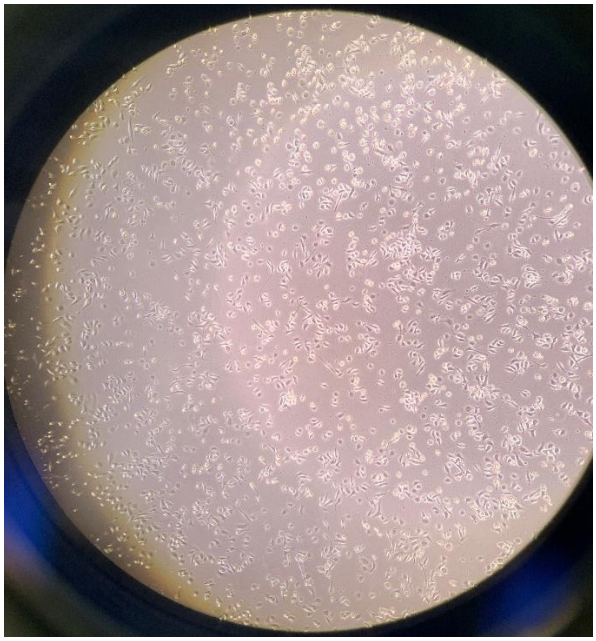

*Figure 14: Differentiated macrophages after 6 days in DMEM and L-cell before induction for experiment. Magnification X10.*

The L-cell was obtained thanks to the seeding of the L-929 cell line (Figure 14). My L-929 cells were awoken on days 3, and 5 and they reached perfect confluency on day 11 (Figure 15c). I followed protocol to then plate them to obtain the L-cell for posterior macrophage differentiations. The macrophages we obtained using the L-cell can be appreciated in figure 14.

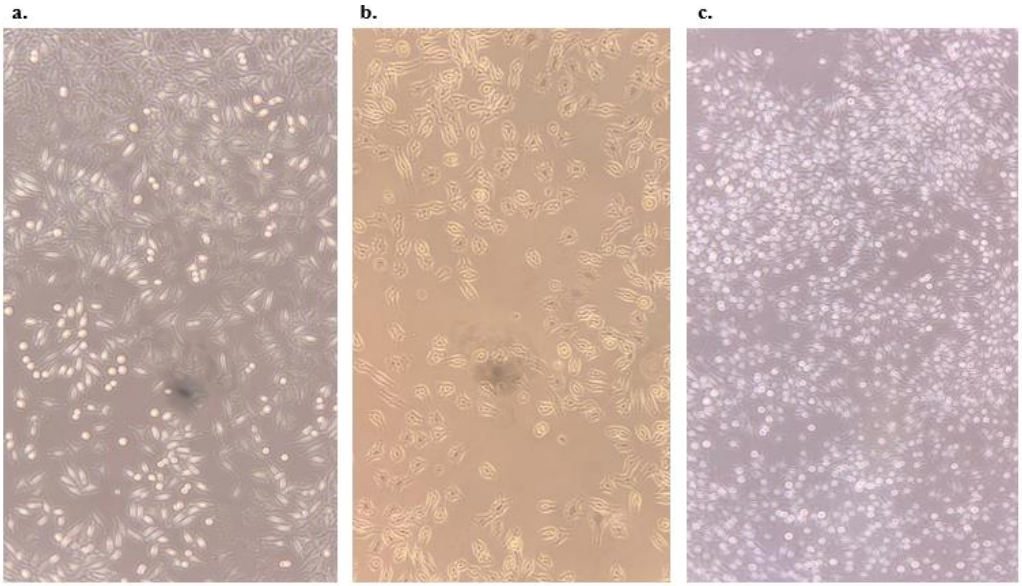


*Figure 15: Figure 14: L-929 cells seeding on days 3 (a), 5(b) and 11(c) before plating for obtaining of M-csf. Magnification X40.*

Once we have the macrophages perfectly differentiated (Figure 14), we proceeded with the gene expression analysis. While we checked for biomarkers of M1 and M2 pathways, at the same time we used a specific primer against the *Slc7a7* gene to be able to back up that the knock-out animals did indeed have the gene knocked out and that the control mice were expressing $y^+$LAT1 as normal. Once this was validated, we could move on to analysing the other results.

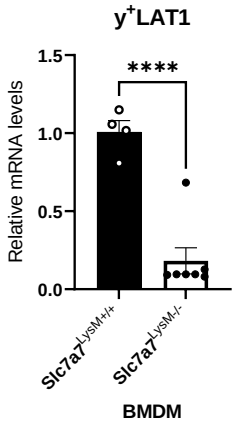


*Figure 16: Depletion of Slc7a7 in bone marrow derived macrophages calculated with relative mRNA expression to b-actin housekeeping gene. Relative quantity of Slc7a7 (2-ΔCT = $2^{(CTbactin - Ctranspoterr)}$) were calculated from corrected CT values. ****pvalue<0.0001. Data represents mean +/- SEM. Graph was made using GraphPad Prism.*

As mentioned previously, macrophages are activated by different stimuli towards two different pathways (Figure 4). The M1 pathway is anti-inflammatory and expresses anti-inflammatory cytokines and the NOS enzyme to differentiate. We induced this pathway with LPS which is a bacterial Lypopolysaccharide which acts as a stimulus for the macrophage to kill and express these biomarkers. The M2 pathway, in turn is anti-inflammatory and is induced through IL-4 which is what we used to induce it. It expresses anti-inflammatory cytokines such as TGF-β as well as the Arg1 enzyme which catalyses the activation towards M2 macrophages. The macrophages were induced for 1 hour before extracting the RNA and doing the qPCR analysis. This experiment had previously been done with an induction time of 24 hours but it was thought that the macrophages being in such a low concentration of arginine for so long could alter the transcription of the genes in some way, so it was decided that I repeat it for a time of 1 hour.

### 4.3.1 M1 Biomarkers analysis

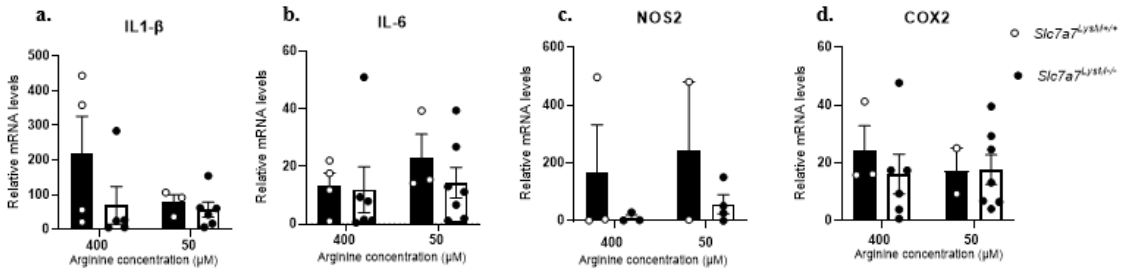


*Figure 17: Relative mRNA levels in arbitrary units of M1 biomarkers induced with 1:1000 LPS in concentrations of 400uM and 50uM of arginine. Comparison between wild type mice (black bars) and LysM knock out mice (white bars). A: mRNA levels of IL-1β, B: mRNA levels of IL-6, C: mRNA levels of NOS2, D: mRNA levels of COX2. Data represents mean +/- SEM. Graphs made using GraphPad Prism.*

The different concentrations of arginine are to mimic an environment of hyperarginemia (400μM), a normal mouse environment (50μM) and an LPI environment of hypoarginemia (20μM). In the M1 analysis of biomarkers the results for the 20μM environment are lacking due to manipulation errors in which the results for the basal control were not viable so I couldn't perform an analysis on this condition (Figure 17).

No statistical differences can be appreciated between genotypes of either of the biomarkers. There is a very high error margin and some lack of samples that make these results inconclusive.

### 4.3.2 M2 Biomarkers analysis

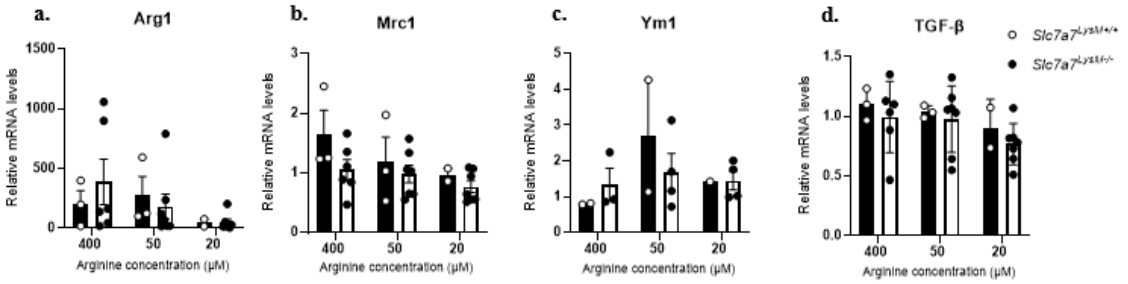


*Figure 18: Relative mRNA levels in arbitrary units of M2 biomarkers induced with 1:1000 IL4 in concentrations of 400uM, 50uM and 20uM of arginine. A: mRNA levels of Arg1, B: mRNA levels of Mrc1, C: mRNA levels of Ym1, D: mRNA levels of TGF-β. Data represents mean +/- SEM. Graphs made using GraphPad Prism.*

In the analysis of M2 biomarkers all three conditions of arginine were compared after the IL-4 induction to the M2 pathway.

No statistical differences can be observed between the genotypes of the expression of either of these biomarkers. The error margin and lack of some samples make these results inconclusive (Figure 18).

# 5. Discussions

## 5.1 Validation of y+LAT1 deletion in LPI model

To eliminate the expression of $y^+$LAT1 in all of the cells of the mouse, a tamoxifen inducible model was designed in which loxP sites were flanking exons 3 and 4 of the *Slc7a7* gene, joined with the Cre recombinase under the UBC promoter. The expression of the recombinase was induced by tamoxifen which ultimately eliminated the expression of *Slc7a7*. This model gives rise to the only viable animal model with Lysinuric Protein Intolerance.

Tamoxifen induction could not have an efficiency of 100%, so it was important to check at a protein level the complete elimination of the transporter (Figure 9).

Through these results we can conclude that all the animals that were supposed to be knocked out, were indeed lacking the expression of $y^+$LAT1. So, in this case, the efficiency of depletions was almost 100%.

## 5.2 Analysis of hepcidin regulation in liver of LysM model

Previous studies suggested that the lack of expression of $y^+$LAT1 in macrophages could be at the base of these complications in the disease. In the animal model in which

we eliminate $y^+$LAT1 in the myeloid cell line (LysM-Cre model), results from the lab showed no major complications compared to the full LPI mouse model. This leads us to believe that it's not the lack of the transporter in macrophages that cause immune complications but more so the LPI environment that arises from the malabsorption and reabsorption in LPI patients (hyperammonemia and hypoarginemia).

In figure 11a you can clearly see no statistical differences between genotypes in *Hamp1* to begin with. Aside from that you also see neither a statistical difference nor a tendency in the regulatory genes IL1-β and IL6 (Figure 11b and 11c), two main inflammatory genes that could trigger hepcidin expression. This seems to make sense, because if hepcidin is not over expressed then the regulatory genes shouldn't be expected to be higher in the knock-out lysozyme mouse. These results corroborate with the hypothesis that this animal model does not suffer any major immune complications if not surrounded with an LPI environment.

5.3 Analysis of hepcidin regulation in liver of CreUBC model

In this animal model, previous results had clearly shown that hepcidin in plasma was expressed more in LPI mice than in controls (Figure 7 from background). So, in figure 12 we expected to see a clear statistical difference in the *Slc7a7* deficient mice expressing higher levels. You can appreciate an obvious tendency but statistically there are no significant differences. If this experiment could be repeated with a higher number of animals, I am confident that the results would shower higher expression in the LPI mouse.

The experiment was repeated with another batch of animals to increase the sample quantity (n) but unfortunately there was no consistency between the two batches, and I was not able to join them together to reach a conclusion.

The analysis of hepcidin regulatory cytokines such as IL-6, IL1-β and the BMP6 protein also show no statistical differences (Figure 13). We need to consider that if the hepcidin levels did not show statistical differences even if it did show a tendency, it was also unlikely that the regulatory elements would show a statistical difference, they may have only showed tendencies. IL1-β and IL-6 (Figure 13b and 13c) show tendencies as well of higher expression in LPI mice, with IL-6 showing a close value to being statistically higher in LPI mouse, but this result is not viable as we only used 2 knock out mice.

What we expected to see was that one of the regulatory elements be expressed more in the LPI mice as that would confirm what is regulating the high levels of hepcidin in LPI mice. This experiment would need to be repeated with a higher number of samples to confirm any hypotheses.

### 5.4 M1 and M2 biomarkers analysis in BMDM

The depletion of $y^+$LAT1 in the LysM model of BMDM in the animals that we analysed was almost complete, as it was expected (Figure 16). This was necessary to analyse any further results.

Due to the many immune complications associated with LPI, the main affected immune cell to look at is more logically the macrophage, being one of the cells that expresses the $y^+$LAT1 transporter. This is due to its importance in an immune response to different stimuli, and the major role arginine plays in its activation, being that arginine levels in LPI patients drastically change.

It was expected to see that in an LPI environment (low arginine), one of the basic functions of macrophages, being its activation, would be altered in some way.

My analysis shows no significant differences between genotypes in neither any M1 biomarkers nor M2 biomarkers (Figure 17 and 18). A few things to note in these results is that firstly the error margins in each case are noticeably high. Another thing is that in a few conditions there is a lack of samples. There are a few possible reasons as to why this occurred.

Firstly, due to the high quantity of samples, although the induction time was supposed to be 1 hour, by the time I did the RNA extraction on so many samples, its possible that some of the samples were left for more than 1 hour. This could have modified the expression in some of the samples.

Another thing I explored was the quality of the RNA of these samples. They showed a rather low 260/230 ratio. The ratio needs to be equal to 2 or more to be considered pure. In being such a low value, this could indicate contamination by phenols.

Although I repeated this experiment, these circumstances mean that these results are not exceptionally reliable. It would need to be repeated with a higher number of samples for both wild type and knock out, with more caution to the time spent in the induction and trying to perfect the RNA extraction to avoid contamination in the future.

I am confident that if this experiment were to be repeated, a logical conclusion could be taken from the results.

# 6. Conclusions

- Induction on the mouse model with tamoxifen produces the almost complete depletion of Slc7a7 transporter and provides a viable and reproducible LPI animal model.

- There are no differences in the expression of Hamp1 or its regulatory genes in the LysM mouse model, concluding that it should be the LPI environment that affects macrophages in LPI and not the lack of the transporter itself.

- No statistically significant differences are observed in hepcidin regulatory genes in the CreUBC animal model.

- The expression of M1 and M2 biomarkers in bone marrow derived macrophages were not conclusive.

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# 8. Appendixes

## 8.1 Protocols

**Protocol for membrane protein extraction**

**Materials and reagents:**

- Membrane protein lysis buffer (25mM Hepes, 4mM EDTA and 250mM sucrose)
- Flat head 2mL Eppendorf tubes?
- Ceramic balls?
- Protease inhibitor (Cocktail set III)
- Ultracentrifuge tubes
- TLA-55 Rotor
- 25G syringe

**Process**

- Place half the kidney in the flat head tubes with 3 ceramic balls and 1mL of membrane lysis buffer containing the correct dilution of protease inhibitor.
- Homogenize for 2 pulses of maximum speed for 25 seconds.
- In the meantime leave the table centrifuge in the lab to be cooling down to 4 degrees because once you have homogenized, centrifuge for 10 minutes at 10000rpms at 4 degrees.
- Once centrifuged, take out the supernatant with a pipette and transfer to a special ultracentrifuge tube in ice.
- Equalize the samples before using the ultracentrifuge. This can be done by weighing them and if there is a higher difference of weight of more than 0.009g per tube, add membrane lysis buffer to lighter samples.
- Ultracentrifuge, placing samples as you've equalized them in the TLA-55 rotor, for 1 hour 55000 G.
- Once done, get rid of supernatant with a micropipette and add in 100µL of lysis buffer to each sample.
- Use a syringe to resuspend and homogenize the sample. It must have no lumps or pieces.

## Protocol for protein evaluation with Pierce kit

### Materials and reagents

- BSA Pierce kit
- 96 well plate

### Process:

- Prepare a 96 well plate with the right BCA concentrations as per the following figure as an example for layout:

| | | |
|---|---|---|
| 0 x2 (5µL buffer) | Sample 1 | Sample 1 |
| 2 x2 (4µL BSA 0.5ug/ µL) | Sample 2 | Sample 2 |
| 5 x2 (10µL BSA 0.5ug/ µL) | Sample 3 | Sample 3 |
| 10 x2 (5µL BSA 2ug/ µL) | … | … |
| 15 x2 (7.5µL BSA 2ug/ µL) | … | … |
| 20 x2 (10µL BSA 2ug/ µL) | … | … |

- Make sure to duplicate each sample.
- Add 2µL of samples in different wells, also duplicated.
- Add 200 µL of the Pierce buffer A and a 1:50 dilution of buffer B of the PIERCE kit per well.
- Put lid on 96 well plate and put in 37ºC incubator for 30 minutes.
- Read absorbance

## Protocol for SDS-Page electrophoresis gel

### Reagents

- Laemmli sample buffer (LSB 1x)
- SDS 10%
- Bis acrylamide 30%
- Resolving buffer (pH=8.8)
- Stacking buffer (pH=6)
- APS (Ammonium Persulfate)
- TEMED (Tetramethylethylenediamine)
- Resolving buffer 1X

### Process

- Prepare samples by adding correct calculation for 30µg of protein, with 7.5µL of LSB and up to 30µL of water.
- Prepare the resolving gel. For two gels: 7.9mL H2O, 6.7mL 30% Bis-acrylamide, 5mL resolving buffer, 200µL SDS 10%, 200µL APS and 20µL TEMED.
- Pour the gel leaving around 2cm below the bottom of the comb.
- Pour in isopropanol to level out gel immediately.
- Prepare stacking gel. For two gels: 5.6mL H2O, 1.7mL Bis-acrylamide, 2.5mL stacking buffer pH 6, 100µL SDS 10%, 100µL APS, 10µL TEMED.
- Pour on top of solidified resolving buffer and add comb in straight away.
- Once gels made, clamp the gels into the apparatus and fill up with resolving buffer (1X).
- Load 30µL of sample in each well and 10µL of molecular weight marker.
- Connect at 100V until the samples have reached the resolving gel and then 150V until the 26KB band has left the gel.

## Protocol for membrane transfer

### Materials and reagents

- Transfer membranes
- Transfer buffer
- Cassettes
- Sponges
- Watman paper

### Process

- Open cassettes and in transfer buffer and make sure to wet membranes and sponges so that nothing is dry.
- Create the sandwich (Sponge, watman paper, gel, membrane, watman paper, sponge).
- Close the cassette tightly so that there are no bubbles forming inside.
- Gel must be placed the opposite way around if you want the transfer to be as the samples were loaded.
- Place in the apparatus with a block of ice at 250mA for 90 minutes.

## Protocol for membrane incubation

### Reagents

- Powdered milk
- PBS
- $y^+$LAT1 antibody (1:750)
- β-actin antibody (1:10000)
- PBS-Tween 0.1%
- Donkey anti-mouse antibody
- Donkey anti-rabbit antibody

### Process

- Block membranes with milk at 5% in PBS (5g of milk in 100mL of PBS) for 1 hour in agitation.
- Cut membrane under the 70kDa marker and place the top cut in $y^+$LAT1 antibody (falcon tube against the wall) and the lower half of the membrane in the β-actin antibody falcon tube.
- Leave overnight at 4ºC on roller.
- Do 3 10 minute washes with PBS-Tween 0.1%.
- Prepare secondary antibodies (β-actin: 2µL in 25mL of PBS and milk 1%, $y^+$LAT1: 4µL in 25mL PBS and milk 1%).
- Leave top membranes in $y^+$LAT1 secondary antibody and lower membranes in β-actin secondary antibody for 1 hour in agitation.
- Wash 3 times for 10 minutes with PBS-Tween 0.1%.

## Protocol for revelation of western blot

### Materials and reagents

- ECL reagents A and B
- Hyperfilms
- Case

### Process

- Put 500µL of reagent A and 500µL of reagent B in an Eppendorf and mix.
- Place membrane between Western blot case and do not let it dry out.
- Add the 1mL per membrane of the reagents onto the membrane evenly.
- Close the plastic and wait for 1 minute.
- Using paper, push down carefully to get rid of any excess liquid between the plastic.
- Use hyperfilms to reveal.
- Always make a bend in top right corner of each film.

## Protocol for RNA extraction in tissues

### Materials and reagents

- Flat head Eppendorf
- Screw on lids
- Ceramic beads
- Trizol reagent
- Chloroform
- Ethanol
- PureLink RNA Mini Kit

### Process

- Add 1mL of Trizol into a flat headed Eppendorf with 3 beads and add tissue.
- Homogenize and make sure it is mixed well and leave for 5 minutes to get lysed.
- Add 200µL of chloroform per every 1mL of Trizol used originally and mix thoroughly by shaking until it is a baby pink colour.
- Centrifuge for 15min at 4ºC at 12000rpm. Everything else is done at room temperature.
- Carefully take out with a pipette the tissue that's a clear aqueous phase layer on the top and put into another Eppendorf. We can move onto the RNA purification step using PureLink RNA Mini Kit.
- Add 300µL of ethanol (70%) and mix with the vortex.
- Pass sample through the column centrifuging 15s at 12000rpm at room temperatura. Make sure you do this step in 700µL tandems. If the sample does not fit do a separate centrifuge.
- Add 700µL of wash buffer I, centrifuge for 15s at 12000rpm, discard flow-through and change the column.
- Add 500µL of wash buffer II with ethanol and discard flow throw. Repeat this step twice.
- Let the column dry by centrifuging for one minute and put in the recovery tube.
- Add the RNAse free water in the correct quantity to elute your RNA. For tissues we use 100µL.
- RNA can either be stored at -20ºC or -80ºC for long periods of time or kept on ice for immediate use.

## Protocol for lysis of cells for RNA extraction

### Materials and reagents

- Lysis Buffer
- DTT
- PBS 1X
- Scraper
- 25G syringe

### Process

- Prepare lysis buffer. We use 300µL per tube and have a 1% Dilution of DTT.
- Suck out the media.
- Wash each place with PBS 1X 3 times.
- Add in 300µL of the prepared lysis buffer with DTT.
- Scrape the plate vigorously with scraper.
- With syringe do up and down before adding to Eppendorfs.
- The RNA extraction is done using the PureLink RNA mini kit using the protocol above.

## Protocol for generation of bone marrow derived macrophages

### Material and reagents

- RBC lysis (R&D systems, 10x) at a 1x concentration
- PBS- Penicillin/Streptomycin (P/S) 1%
- DMEM 1X+FBS (10%)+ P/S 1%
- 40µm filter strainer
- Falcon tubes

### Process

- Once the mouse has been sacrificed, the bones must be peeled and they extract the femur and tibia.
- They make sure to cut a hole in each of these bones and put them in 0.5mL epi with wholes cut in the bottom with a needle which in turn will be put in 1.5mL epi. They make sure to put 2 bones per epi.
- Centrifuge these so that the bone marrow is flushed to the bottom of the 1.5mL epi and now we can discard the 0.5mL epi with the empty bones.
- Resuspend the bone marrow with 1mL of PBS and pass it through a strainer of 40µm in size to a 50mL falcon tube. All 4 bones per animal go into the same tube.
- Centrifuge the cells at 1250rpm for 5 minutes at 4ºC.
- Perform the erythrolysis on the pellet. For this, resuspend the pellet in the lysis buffer.
- Leave it for one minute at room temperature and then add 25mL of PBS+P/S to stop the lysis.
- Centrifuge the cells again at 1250 for 5 minutes at 4ºC making sure the pellet is white.
- Wash them with another 25mL of PBS+S/P and centrifuge one more time.
- Resuspend the cells in 15mL of DMEM and plate them.
- We plated 3 plates per animal for the three conditions. Each plate will contain:
    - 5mL of BM in DMEM (+P/S)
    - 3mL of FBS 10%
    - 9mL of L-cell
    - 13mL of DMEM(+P/S)
- Leave untouched for 6 days.

## Protocol for Obtaining of L-cell

### Material and reagents

- DMEM 1x+10% FBS+1% P/S
- PBS
- 500mL filter unit 0.2µm

### Process

- Defrost the cells at room temperature.
- Add them to a 15mL falcon with complete DMEM.
- Centrifuge the cells for 5 minutes at 1250rpm and discard the supernatant without touching the cells.
- Resuspend the cells with 10mL of DMEM and plate them with around 15mL in a 15cm plate.
- Incubate for 6 days.
- Do 2 passes before being able to collect M-csf (Suck out medium, wash with PBS, add more medium to plate, scrape and replate in different dilutions).
- They can’t reach a higher confluency than 80%.
- Plate 500.000 cells in 40mL of medium, in as many plates as you can.
- Leave for 7 days.
- Collect supernatant, filtering with 500mL filter unit 0.2µm.

**Protocol for induction of BMDM**

**Materials and reagents**

- Scraper
- Invitrogen countless plates
- 6 well plates
- SILAC DMEM
- Complete DMEM
- Arginine 50mM

**Process**

- Scrape plates and put the cells in medium in falcons. We put them homogenously in 2 separate falcons as there should be around 80mL of medium at this point.
- Take 10µL of cells of each animals and use the Invitrogen countless to count the cells.
- Plate the right amount to have 2 million cells per plate.
- Each animal must have 2 six well plates, for 3 different conditions of arginine and 3 different inductions.
- Leave cells to seed for 2 hours.
- Prepare the arginine mediums:
    - 400uM of arginine: This is simply the complete DMEM with added 10% FBS and 1% P/S.
    - 50uM of arginine: SILAC DMEM in a 50mL falcon and add in 50µL of the 50mM Stock of arginine.
    - 20uM of arginine: SILAC DMEM in a 50mL falcon and add in 20µL of the 50mM stock of arginine.

*We´re preparing 50mL in this case because there are 6 wells of SILAC medium per animal and a total of 6 animals, and we put 2mL per well=18x2=36, make more to be sure.

- Change the medium by sucking out the extra medium and we add in 2mL of the corresponding medium per well.
- For induction plates, do a 1:1000 dilution of LPS and IL4. For every 2mL well we will add in 2µL of LPS or IL4.

- After 1 hour I will get the plates and perform RNA extraction as per the protocol mentioned above.

## 8.2 Thermocycler settings

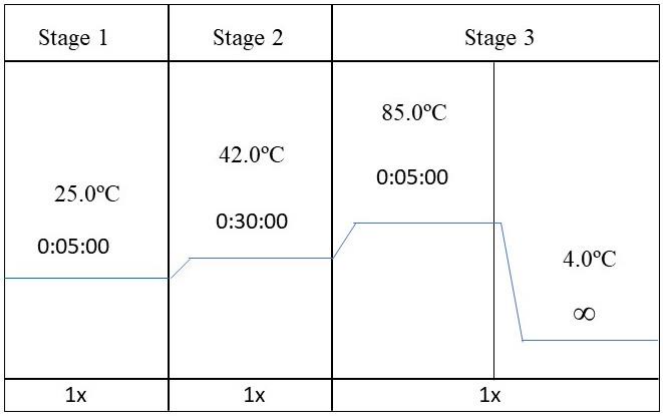


*Figure 19: Thermocycler settings for reverse transcription step in qPCR analysis.*