

Faculté des sciences

The effect of aging on liver inflammation: *Nothobranchius furzeri* as a promising model

Auteur : Kyle Deflandre

Promotrice : Prof. Melissa PAGE

Doctorante : Hélène SCHRAVERUS

Lectrices : Prof. Cathy DEBIER et
Prof. Isabelle DONNAY

Année académique 2023-2024

Mémoire de fin d'études présenté en vue de l'obtention du diplôme de
Biochimie et biologie moléculaire et cellulaire à finalité spécialisée

Abstract (French version)

Le vieillissement est défini comme l'accumulation naturelle et progressive de changements physiologiques au fil du temps, conduisant à des maladies et inévitablement à la mort. Avec les améliorations actuelles dans le système de soins de santé, la durée de vie humaine moyenne a considérablement augmenté. Cependant, le vieillissement avancé est corrélé à l'apparition de plusieurs maladies telles que le syndrome métabolique, les cancers, les maladies cardiovasculaires et les maladies neurodégénératives.

Le vieillissement a été défini avec 12 caractéristiques, dont l'une récemment ajoutée est l'inflammation chronique. Bien que l'inflammation soit un mécanisme de défense naturel, si elle n'est pas résolue, elle peut conduire à une inflammation chronique qui peut avoir un impact négatif sur les cellules immunitaires. Un nouveau terme est apparu pour décrire cette inflammation chronique liée à l'âge : l'inflamming, qui est défini comme un dérèglement de la réponse inflammatoire caractérisée par une production accrue de cytokines pro-inflammatoires accompagnée d'une production réduite de cytokines anti-inflammatoires. Par exemple, les cytokines pro-inflammatoires clés impliquées dans l'inflammation comprennent TNF- α , IL-8, IL-1 β , IL-6 et TLR2, tandis que les cytokines anti-inflammatoires clés comprennent IL-10, APOA-1 et SIRT-1.

Nothobranchius furzeri est un modèle animal relativement récent pour examiner les mécanismes moléculaires et cellulaires sous-jacents au vieillissement. Des résultats ont commencé à émerger montrant la présence de marqueurs d'inflamming dans le système optique de *N. furzeri*. Nous souhaitons explorer davantage si l'expression des cytokines mentionnées ci-dessus est affectée par le vieillissement avancé dans d'autres tissus de *N. furzeri*. Très peu d'éléments montrent que l'âge modifie l'expression de ces gènes dans des homogénats de tissus cérébraux entiers. L'objectif de cette thèse est de déterminer si cela est également vrai ou non pour le tissu hépatique, un autre tissu sensible à l'inflammation liée à l'âge.

Des expériences de PCR en temps réel ont été menées sur huit gènes associés à l'inflammation (IL-10, TLR2, APOA-1, IL-8, TNF- α , IL-1 β , SIRT-1 et IL-6) à travers quatre âges (6, 14, 20 et 26 semaines). Sur les huit gènes, nous avons pu mesurer l'expression génique tout au long du vieillissement pour quatre d'entre eux : deux molécules anti-inflammatoires, IL-10 et APOA-1, ainsi que deux molécules pro-inflammatoires, TLR2 et IL-8.

L'expression d'IL-10 est significativement plus élevée à 14, 20 et 26 semaines par rapport à 6 semaines. En revanche, nous n'avons détecté aucune différence dans l'expression génique d'IL-8, TLR2 et APOA-1 à travers les quatre âges. L'expression génique n'a pas non plus été affectée par le sexe biologique.

Acknowledgements

This thesis was a beautiful journey that would not have been possible without a lot of people.

First, I would like to thank Professor Melissa Page for choosing me over other students to realize my thesis in her team within the BNTE lab. I really think it's a privilege to work here, surrounded with nice people who are eager to search, discover and learn in the scientific domain. I would like to thank Melissa for her guidance, her attention, her enthusiasm, and her trust since the beginning and until the end. I have learned so much here and I will always be grateful.

Then, I would like to thank my PhD mentor Hélène Schraeverus, who followed me during the whole length of this thesis, and it was quite extended thanks to general chemistry 2. She was always there to answer any of my questions or help me if I needed. I started my thesis late, but she was there to help me catch up. We formed a great team, but I had no doubt since we both know Peter Quill is the best superhero!

I would like to thank Ineke Neefs, she taught me everything I had to know to work in the lab, from how to use all the equipment, to the importance of a cleaned hood!

I would like to thank Oksana Savel who was always ready to help if I needed any information for my thesis, or any question concerning the Killifish. I also want to thank Catherine Rasse who helped me a lot for the statistical analysis of my thesis.

Finally, I would like to thank every people I met in the lab, PhD students, technicians, other Master' students, it was truly a pleasure.

Table of content

Abstract (French version)	2
Acknowledgements	3
List of figures	6
List of tables	7
State of art.....	8
1 Aging.....	8
2 Inflammation	10
3 Cytokines – markers of inflammation	15
3.1 Pro-inflammatory cytokines	15
3.1.1 Tumor necrosis factor α (TNF- α):.....	15
3.1.2 Toll-like receptor 2 (TLR2) :	17
3.1.3 Interleukin 1 Beta (IL-1 β) :	18
3.1.4 Interleukin 8 (IL-8) :	18
3.2 Pleiotropic cytokine	18
3.2.1 Interleukin 6 (IL-6) :	18
3.3 Anti-inflammatory cytokines	20
3.3.1 Apolipoprotein A-1 (APOA-1) :	20
3.3.2 Interleukin 10 (IL-10) :	21
3.3.3 Sirtuin 1 (SIRT-1) :	22
4 Inflammaging.....	23
5 Liver	24
5.1 Introduction and liver anatomy.....	24
5.2 Liver multiple roles	25
5.3 Inflammation in liver	26
6 <i>Nothobranchius furzeri</i> model	27
6.1 Description of the model.....	27
6.2 Aging in <i>Nothobranchius furzeri</i>	28
Objectives.....	30
Material and Methods.....	32
1 <i>Nothobranchius furzeri</i> breeding	32
2 Artemia culture	32
3 Experimental Fish	32
4 RNA extraction and quantification.....	33
5 cDNA synthesis	33

6	RT-qPCR	34
6.1	General information	34
6.2	Primer selection and design	34
6.3	Primer optimization	36
6.4	Reference genes	36
6.5	Gene expression across age	37
6.6	Data treatment and statistical analysis	37
6.6.1	Impact of age on gene expression	38
6.6.2	Impact of sex on gene expression	38
	Results and discussion	40
1	Results	40
1.1	Primer optimization	40
1.1.1	Genes of Interest (GOI)	40
1.1.2	Reference genes	42
1.1.3	Reference genes selection	43
1.2	Gene expression across aging	43
1.2.1	Relative gene expression	44
2	Discussion	48
	Conclusion and perspectives	50
	References:	52
	Annex	56

List of figures

Figure 1: The current hallmarks of aging-----	9
Figure 2: Pathogen detection by macrophages through their PRRs-----	10
Figure 3: The different steps and actors of inflammation and its resolution-----	11
Figure 4: Pathways of inflammation mediators-----	12
Figure 5: Actors and steps of inflammation-----	13
Figure 6: Innate and adaptive system graph response over time-----	13
Figure 7: Activity of immune cells through time-----	13
Figure 8: TNF signaling pathways-----	16
Figure 9: TLR2 link to cholesterol homeostasis-----	17
Figure 10: IL-6 signaling pathways-----	19
Figure 11: APOA-1 implication in cholesterol homeostasis-----	21
Figure 12: IL-10 signaling pathway-----	22
Figure 13: Hepatic Lobule-----	24
Figure 14: Liver functions-----	25
Figure 15: Lifecycle of wild <i>N. furzeri</i> -----	27
Figure 16: GRZ and MZM strains of males <i>N. furzeri</i> -----	28
Figure 17: Male and female <i>N. furzeri</i> at several ages (GRZ strain) -----	28
Figure 18: Reference gene stability using the GeNorm algorithm-----	37
Figure 19: IL-10, TLR2, APOA-1 and IL-8 primer pair ratio standard curves-----	41
Figure 20: EF1 α , INSR, ACTB, TBP, HPRT and GAPDH primer pair ratio standard curves-----	42
Figure 21: IL-10 relative expression across aging-----	44
Figure 22: IL-10 relative quantification of males compared to females-----	44
Figure 23: APOA-1 relative expression across aging-----	45
Figure 24: APOA-1 relative quantification of males compared to females-----	45
Figure 25: IL-8 relative expression across aging-----	46
Figure 26: IL-8 relative quantification of males compared to females-----	46
Figure 27: TLR2 relative expression across aging-----	47
Figure 28: TLR2 relative quantification of males compared to females-----	47
Figure 29: gene expression of inflammatory markers in CNS of <i>N. furzeri</i> -----	48

List of tables

Table 1: Synthesis of cDNA, steps and parameters-----	33
Table 2: Stages and parameters for the PCR run-----	34
Table 3: Primers Information-----	35
Table 4: CT mean values for the reference genes across aging-----	43

State of art

1 Aging

Humans are living longer than ever before, and the population of older individuals continues to grow. Epidemiological studies reveal that 11% of the world's population is over 60 years of age with a suggested increase of 22% by 2050 (1). Longer lifespans are, in large part, due to improvements in the health care system. However, advanced age is also positively correlated with the occurrence of several health issues, such as metabolic syndrome, cancers, cardiovascular diseases, and neurodegenerative diseases.

Aging is defined as the natural and progressive accumulation of physiological changes over time, which lead to diseases and inevitably to death (2). Ten years ago, a scientific review outlined nine hallmarks that characterize aging (3). More recently the same authors revisited the hallmarks and now 12 have been proposed (figure 1) (4). These hallmarks have been divided into three categories (primary, antagonistic, integrative) characterizing the role the hallmark has with aging (figure 1). Primary hallmarks include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy. Antagonistic hallmarks include deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence; and finally Integrative hallmarks consist of stem cell exhaustion, altered intercellular communication, chronic inflammation and dysbiosis

Although they are presented as individual hallmarks, they are often not truly isolated from one another, and several hallmarks are indeed related. For example, genomic instability can impact telomere attrition while mitochondrial dysfunction can alter epigenetic alterations.

For these mechanisms or targets to be identified as a hallmark, they must conform to the following three properties:

1. The hallmark should manifest during normal aging;
2. The experimental aggravation of the hallmark should accelerate aging;
3. The experimental improvement of the hallmark should slow aging.

Inflammation and more specifically chronic inflammation was one of the three recently included hallmarks. Previously it had been categorized within altered intercellular communication.

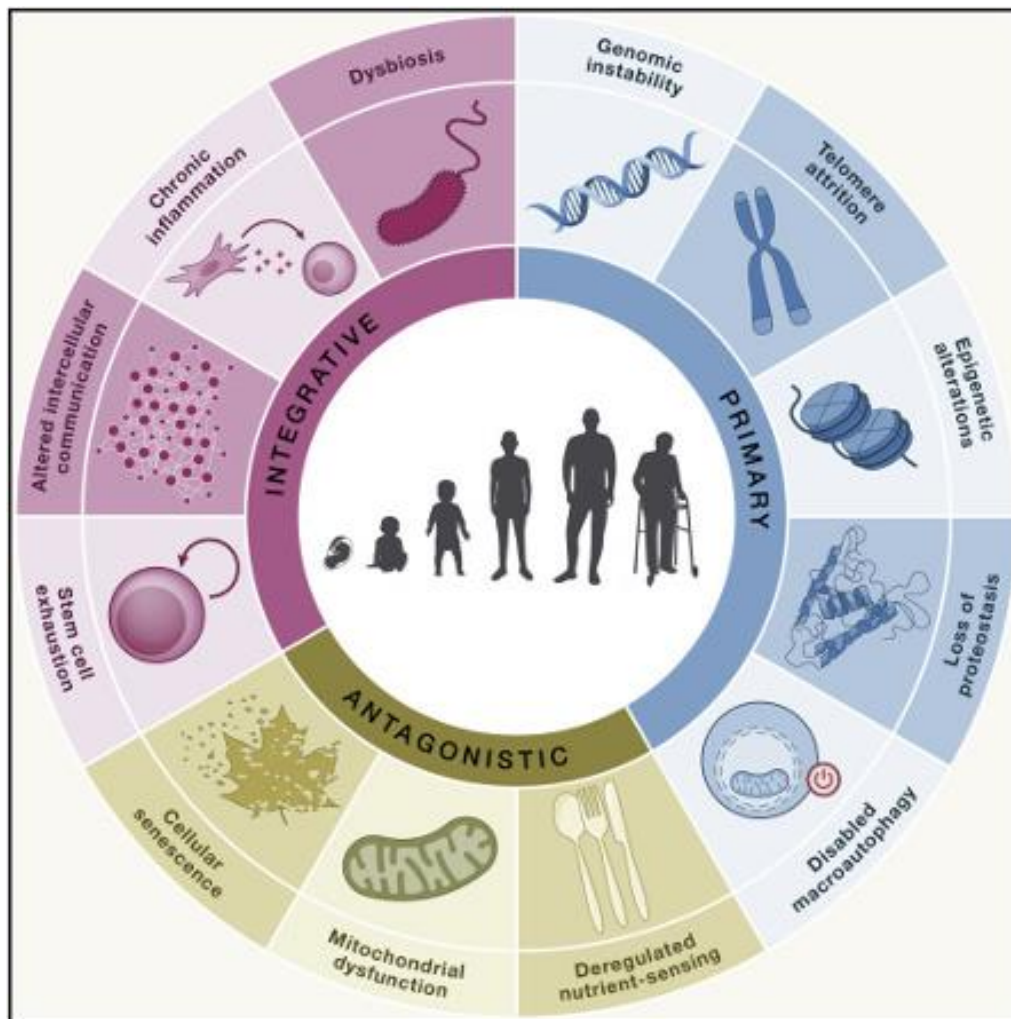


Figure 1: The current hallmarks of aging separated into three major categories: Primary hallmarks considered to be the primary causes of cellular damage; Antagonistic hallmarks considered having compensatory or antagonistic effects in response to cellular damage; Integrative hallmarks considered to be the results of the two other categories, ultimately responsible of the functional decline of aging (4).

2 Inflammation

The first protection our body provides against pathogens are physical barriers such as the skin, mucous membranes, mucous and secretions including saliva and gastric acid. The inflammation mechanism is next in the line for defense. The inflammation response is the major actor of innate immunity whose role is to react quickly but nonspecifically to infections or exogenous materials like allergens or pathogens.

In the case of a pathogen infection, immune cells will recognize pathogen-specific motifs called PAMPs (Pathogen/Protein Associated Molecular Patterns). In a similar way, damaged cells will present specific molecules indicating their condition, these are called DAMPs (Damaged/Danger Associated Molecular Patterns). PAMPs can be inherent components of bacteria, for example LPS (lipopolysaccharide), cell wall sugars or flagellin. DAMPs are normal cellular components present in the wrong location such as uric acid, ATP, cytokine IL-1 α or nuclear proteins which are released intra or extracellularly in response to cell death or tissue injury (5).

Damage or pathogen associated molecular patterns are quickly recognized by pattern recognition receptors (PRRs) present on the surface of many immune cells, including monocytes, macrophages, neutrophils as well as dendritic cells and some lymphocytes (figure 2, 3).

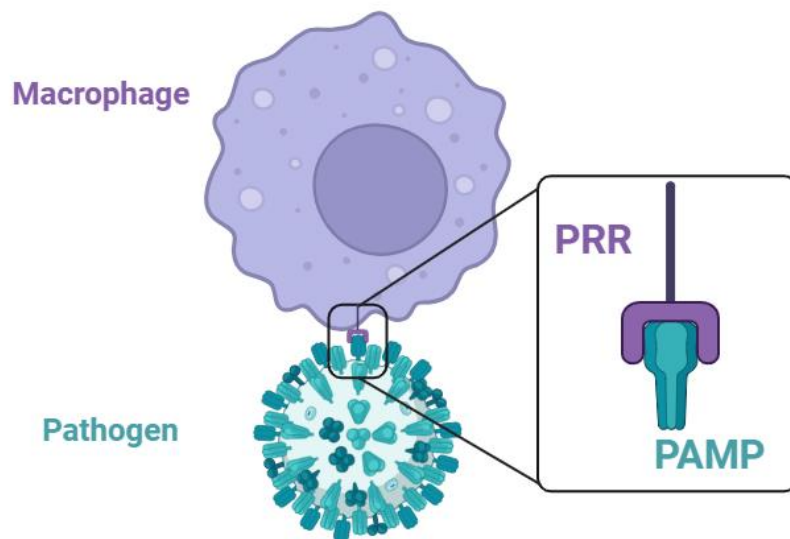


Figure 2: Pathogen detection by pattern recognition receptors (PRRs) of macrophages.

All PRRs are proteins which can recognize a wide variety of exogenous lipids, carbohydrates, peptides, and nucleic acids commonly representative of pathogens but distinct from host molecules. PRRs are divided into five classes depending on their primary function, ligand specificity, structure, composition, and localization. Among those we find the Toll Like Receptor class (TLR - detailed in the *pro-inflammatory cytokines* section) present on all cells but further expressed on host antigen processing cells as they play a critical role in the immune system. Activating a pattern recognition receptor will induce a downstream transcriptional activation and expression of inflammation cytokines (figure 3) (6).

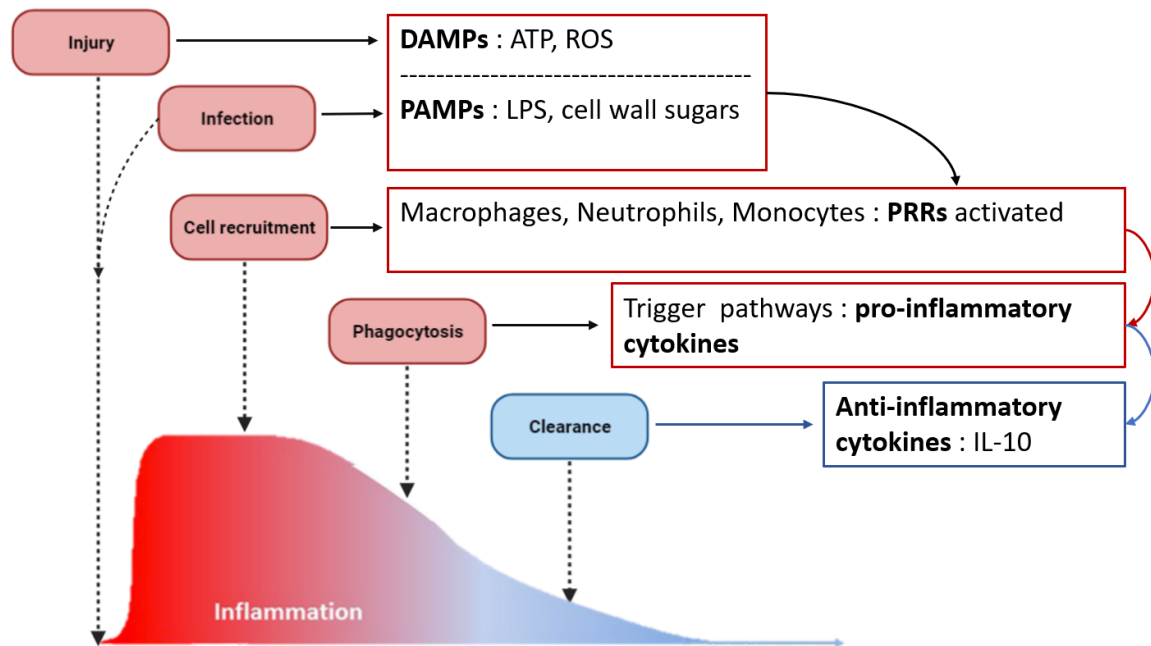


Figure 3: The actors involved in inflammation and its resolution. Modified from Rea et al. (2018).

These immune cells produce highly proinflammatory lipid mediators through three pathways: the cyclooxygenase (COX) pathway, the 5-lipoxygenase pathway (5-LOX) and the cytochrome 450 pathway (CYTP).

COX, 5-LOX and CYTP pathways are part of the arachidonic acid (AA) metabolism. AA is stored in plasma membranes in the form of phospholipids and must first be extracted by phospholipase A2 (PLA2) and released intracellularly. AA can then be metabolized into biological mediators/cytokines (prostaglandins (PGs), leukotrienes (LTs), hydroxyeicosatetraenoids (HETEs)), which are responsible for the four inflammation symptoms: pain, redness, swelling, and heat (figure 4).

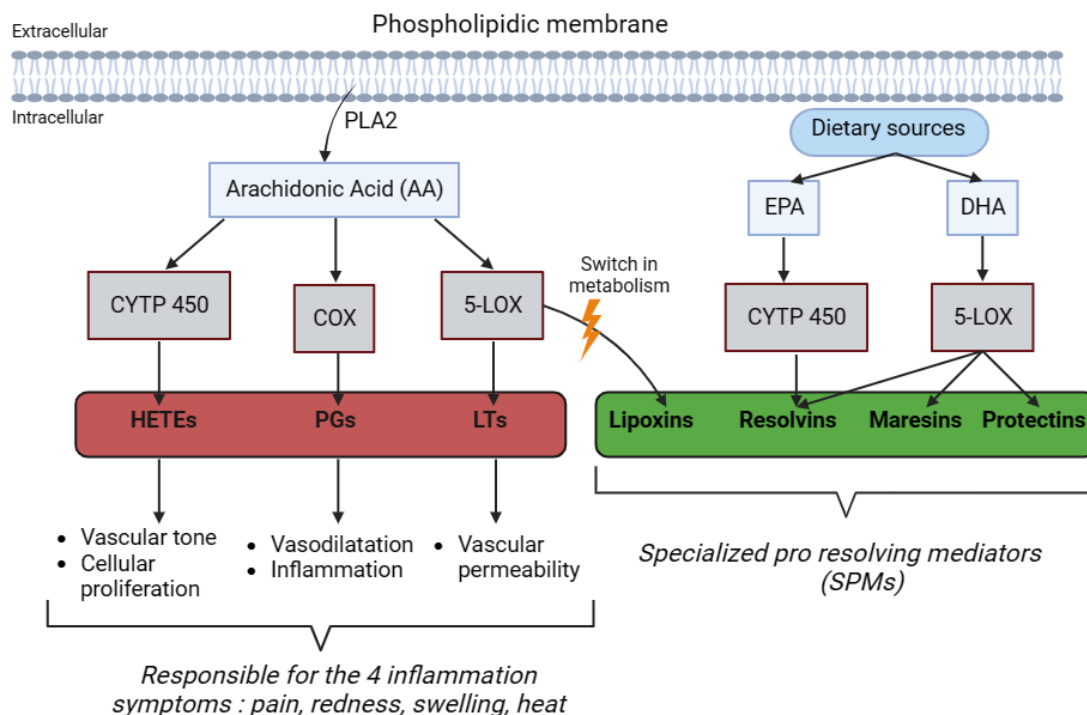


Figure 4: Pathways of inflammation mediators (5,7).

These three pathways have a specific role in the inflammation response. The cytochrome 450 pathway induces vascular tone: vasoconstrictor and vasodilatory effects in blood vessels. This is regulated by the rate of Ca^{2+} on smooth muscular cells. It allows increased blood flow and, consequently, transport of inflammatory molecules to the inflamed area. The COX pathway produces prostaglandins (PGs), which amplify the inflammatory response by enhancing the cytokine cascade, recruiting macrophages associated with inflammation, increasing cytokine expression. Finally, the 5-LOX pathway it induces vascular permeability. This pathway can also produce lipoxins once a switch in metabolism towards resolving inflammation has occurred.

The inflammation response can also be enhanced by increasing vascular permeability due to factors like histamines and chemokines released in blood vessels. In addition to adhesion molecules (integrins and selectins), this allows neutrophils, which act as the first line of defense to migrate to the site of injury as well as phagocytosing pathogens to remove damaged or infected cells. Inflammation is a natural process that must be well regulated to avoid excessive damage. After the acute inflammatory response has controlled the injury, the system returns to immune homeostasis.

The resolution of pro-inflammatory states is controlled by the specialized pro-resolving mediators (SPMs) that possess anti-inflammatory effects. At peak inflammation, a switch in metabolism occurs so that AA will be metabolized through the 5-LOX pathway producing lipoxins (such as SPMs). Other lipid mediators like resolvins, protectins and maresins originating from dietary sources (EPA and DHA, fatty acids mainly present in fatty fish, eggs and dairy products), also act as SPMs (figure 4,5).

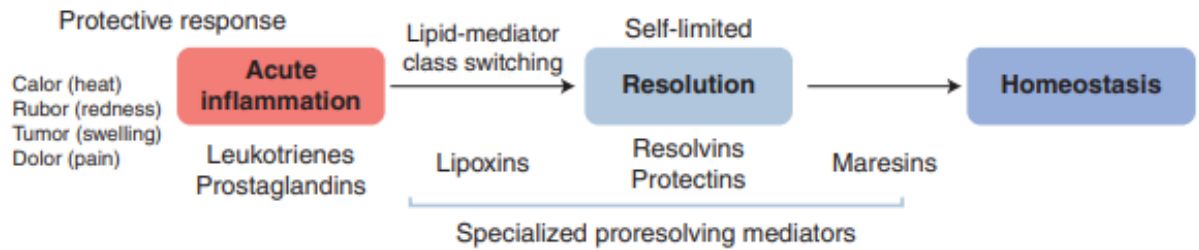


Figure 5: Summary of steps involved in inflammation (7).

Initial inflammation response begins when mediators like PGs and LTs brings neutrophils (first line of defense) through an exudate directly to the infected/damaged tissue. As mentioned before, this step is enhanced by other factors provoking the four symptoms of inflammation. These neutrophils will further induce a response with the recruitment of macrophages which can phagocytize infected/damaged cells (figure 6).

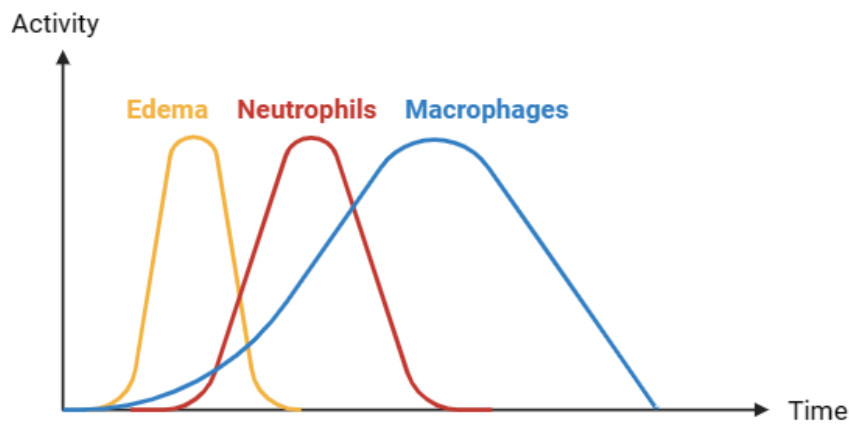


Figure 6: Activity of immune cells over time (7).

Macrophages are the immune cells linking innate to adaptive immunity. The adaptive immunity is activated at a later stage during infection, and its response is slower but highly specific compared to innate immunity (figure 7).

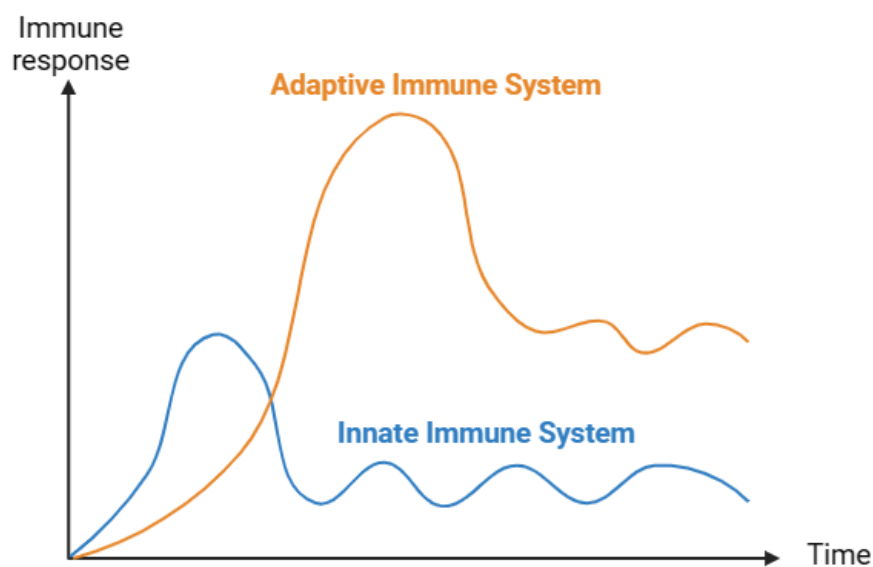


Figure 7: Innate and adaptive system response over time.

Macrophages are classified as antigen presenting cells (APC) and have the capacity to take up microbial components and present them through their Major Histocompatibility Complex (MHC) to B and T lymphocytes, which are adaptive immune cells.

Peak/acute inflammation occurs when multiple neutrophils and macrophages are present at the site of infection. Spatial cell to cell interactions between immune cells will induce the switch in metabolism, from peak inflammation to the resolving phase:

The three classic signs of tissue returning to homeostasis are:

1. Active signals preventing further neutrophil recruitment
2. Enhancement of phagocytosis of apoptotic neutrophils by macrophages
3. PGs and LTs synthesize SPMs for inflammation resolution

In addition, during the resolving phase, monocytes also release anti-inflammatory cytokines (i.e. IL-10). Finally, the presence of soluble receptors of major pro-inflammatory cytokines, like TNF α and IL-1 family cytokines, also inhibits their activity. If the inflammation state persists and is not resolved, inflammation becomes chronic, which can have an adverse effect on health.

3 Cytokines – markers of inflammation

3.1 Pro-inflammatory cytokines

Pro-inflammatory cytokines are essential in the inflammation process as their signaling cascade induces the host defense against pathogens, infections, or injuries.

3.1.1 Tumor necrosis factor α (TNF- α):

Tumor necrosis factor α is believed to be the key regulator of the inflammatory response as it mediates its effect by interacting with two different receptors (TNFR1 and TNFR2). These transmembrane proteins are differentially expressed on cells: TNFR1 is expressed on all cell types whereas TNFR2 is selectively expressed on immune cells and endothelial cells. It is still unclear why TNF- α interacts with these two receptors however, both receptors initiate distinct yet overlapping signal transduction pathways (figure 8) (8). TNFR1 is characterized by its cytoplasmic death domain which can recruit TRADD (TNF- α receptor associated death domain) once TNF- α binds to TNFR1. Through TRADD, TNFR1 can either induce a cascade leading to apoptosis or interact with TRAF2 (TNF receptor associated factor 2) eventually leading to NF- κ B, JNK or MAPK activation. Binding of TNF- α to TNFR2 triggers a signaling cascade through TRAF2 leading to NF- κ B activation. The pro-inflammatory effects caused by TNF- α are mainly due to its ability to activate these NF- κ B transcription factors for all types of cells, leading to the expression of many inflammatory genes (9).

TNF- α , together with other pro-inflammatory molecules such as IL-1 β and IL-6, reveal an age-related upregulation. Indeed, studies showed a progressive exhaustion of naïve T cells pool, replaced by an expansion of a more antigen experienced cytotoxic T cells pool (containing pro-inflammatory cytokines such as TNF- α) in aged humans (from 25 to 101 years) (10). Also, elevated circulating levels of TNF were associated with reduced muscle strength, increased risk of cerebrovascular and cardiovascular events and to a more rapid cognitive decline in the elderly population (10).

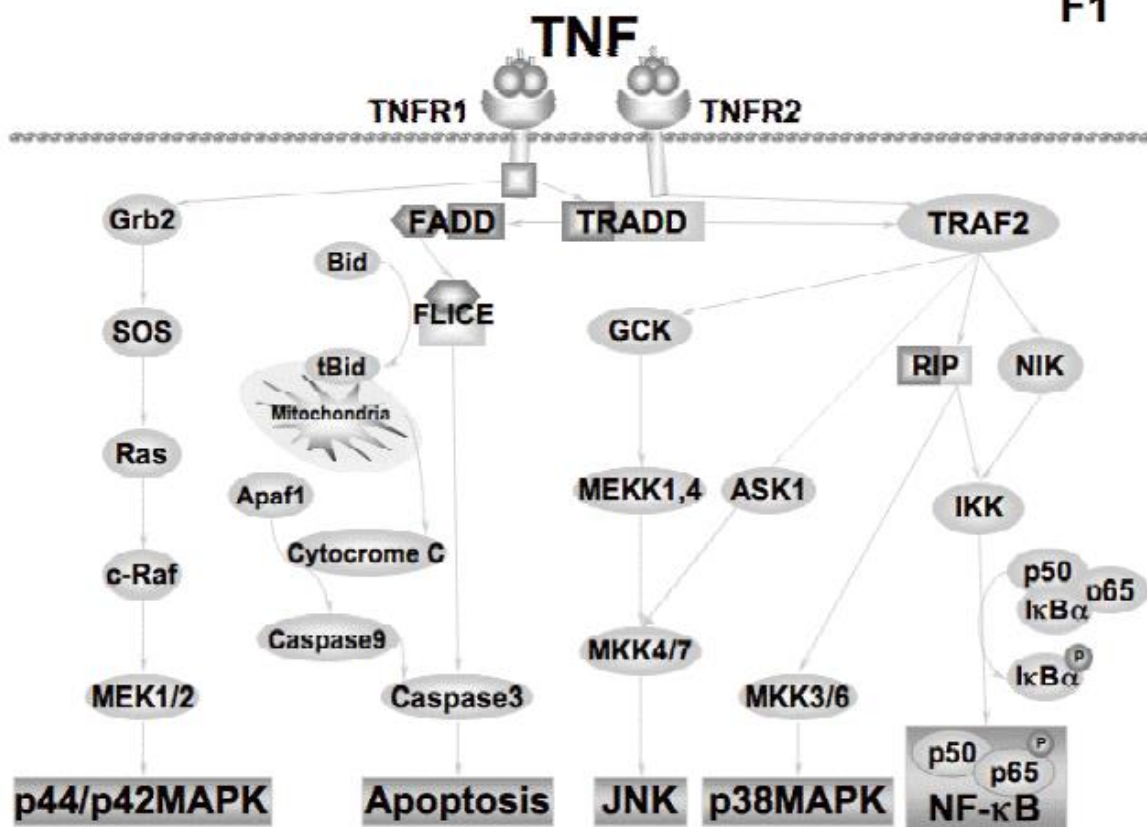


Figure 8: TNF signaling pathways (9).

3.1.2 Toll-like receptor 2 (TLR2) :

TLR2 is part of the pattern recognition pattern family (PRRs) and is a transmembrane glycoprotein localized on the surface of all cell types. It functions by recognizing pathogens or damaged tissue via PAMPs or DAMPs. TLR2 is an immune regulator, its main role is to stimulate phagocytosis and mediate inflammation by triggering a pro-inflammatory signal.

TLR2 is also involved in cholesterol homeostasis. Low density lipoproteins (LDL) inside the artery wall will be modified through either oxidation or aggregation and can act on two levels. It can either be taken up inside macrophages through specific transporters (cholesterol uptake), or it can act as a ligand for macrophage PRRs, including TLR2, and can thereby directly trigger pro-inflammatory signaling pathways (figure 9) (11).

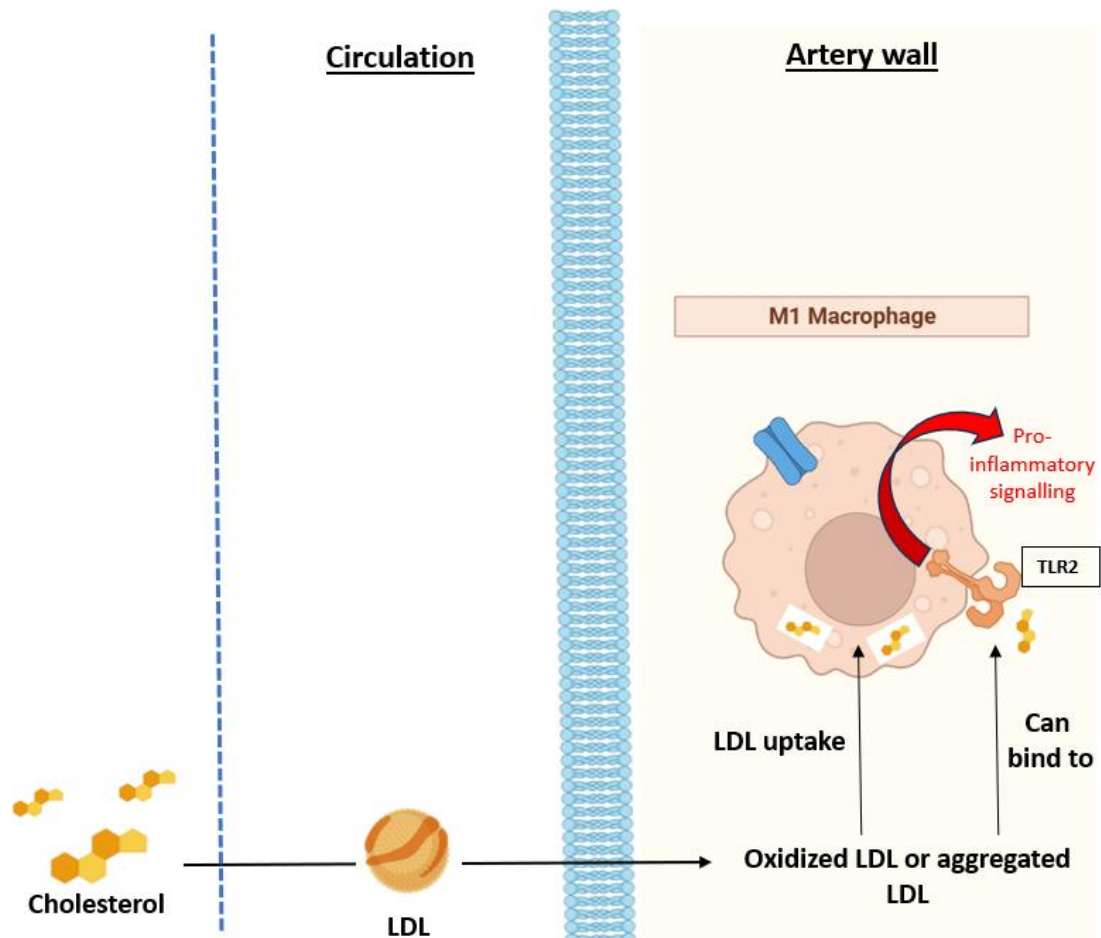


Figure 9: The role of TLR2 in cholesterol homeostasis. Modified from Tall et al., (2015).

3.1.3 Interleukin 1 Beta (IL-1 β) :

The IL-1 gene family contains 11 members. The three major family members are IL-1 α and IL-1 β (also referred to as IL-1), and IL-1 receptor antagonist (IL-1ra).

IL-1 α and IL-1 β exert similar effects by binding to the IL-1 type I receptor. IL-1 α and IL-1 β also bind to IL-1 type II receptor, however this receptor acts as a decoy and is not involved in signal transduction, thereby counterbalancing the inflammatory effects of IL-1 α and IL-1 β . IL-1ra is an endogenous inhibitor of IL-1 α and IL-1 β , and therefore also prevents inflammatory effects by competitively binding to the IL-1 type I receptor without its activation (5,12).

The IL-1 cytokine family are produced by immune cells involved in the immune response such as macrophages, monocytes, fibroblasts, dendritic cells, lymphocytes B and natural killers (NK). IL-1 secretion is induced by microbial products that stimulate toll-like receptors (i.e. LPS) and by certain endogenous triggers, such as uric acid produced during programmed cell death. Both types of activation (exogenous or endogenous) stimulate a cytosolic complex of proteins termed the inflammasome, which activates caspase-1 to enable secretion of IL-1 β (12). Via expression of integrins on leukocytes and endothelial cells, IL-1 β regulates and initiates inflammatory responses (13).

3.1.4 Interleukin 8 (IL-8) :

IL-8 is part of a large family known as chemokines. It has been shown in several studies that IL-8 is at the root of acute inflammation. Indeed, this chemokine is secreted by macrophages to recruit and activate neutrophils (5). Moreover, IL-8 is a promising biomarker and is currently used for many clinical conditions and is being applied for the purpose of rapid diagnosis or as a predictor of prognosis. For example, IL-8 can be used as a biomarker for pulmonary infections, marker of urinary bladder cancer, osteomyelitis, among others (14).

3.2 Pleiotropic cytokine

3.2.1 Interleukin 6 (IL-6) :

IL-6 is referred to as a pleiotropic cytokine due to its ability to function in both pro- and anti-inflammatory processes. On target cells, IL-6 binds and forms a complex with the IL-6 receptor (IL-6R) but does not induce further signaling. The complex needs to interact with the transmembrane protein gp130, which then induces a dimerization with another gp130 to initiate intracellular signaling (figure 10 A). Gp130 is present on the membranes of all cells compared to IL-6R which is only present on a limited number of cells such as hepatocytes, leukocytes, and some epithelial cells. Interestingly there is no detected affinity between IL-6 and gp130. Therefore, as both IL-6R and gp130 are needed, IL-6 signaling can only be triggered on those cells (15).

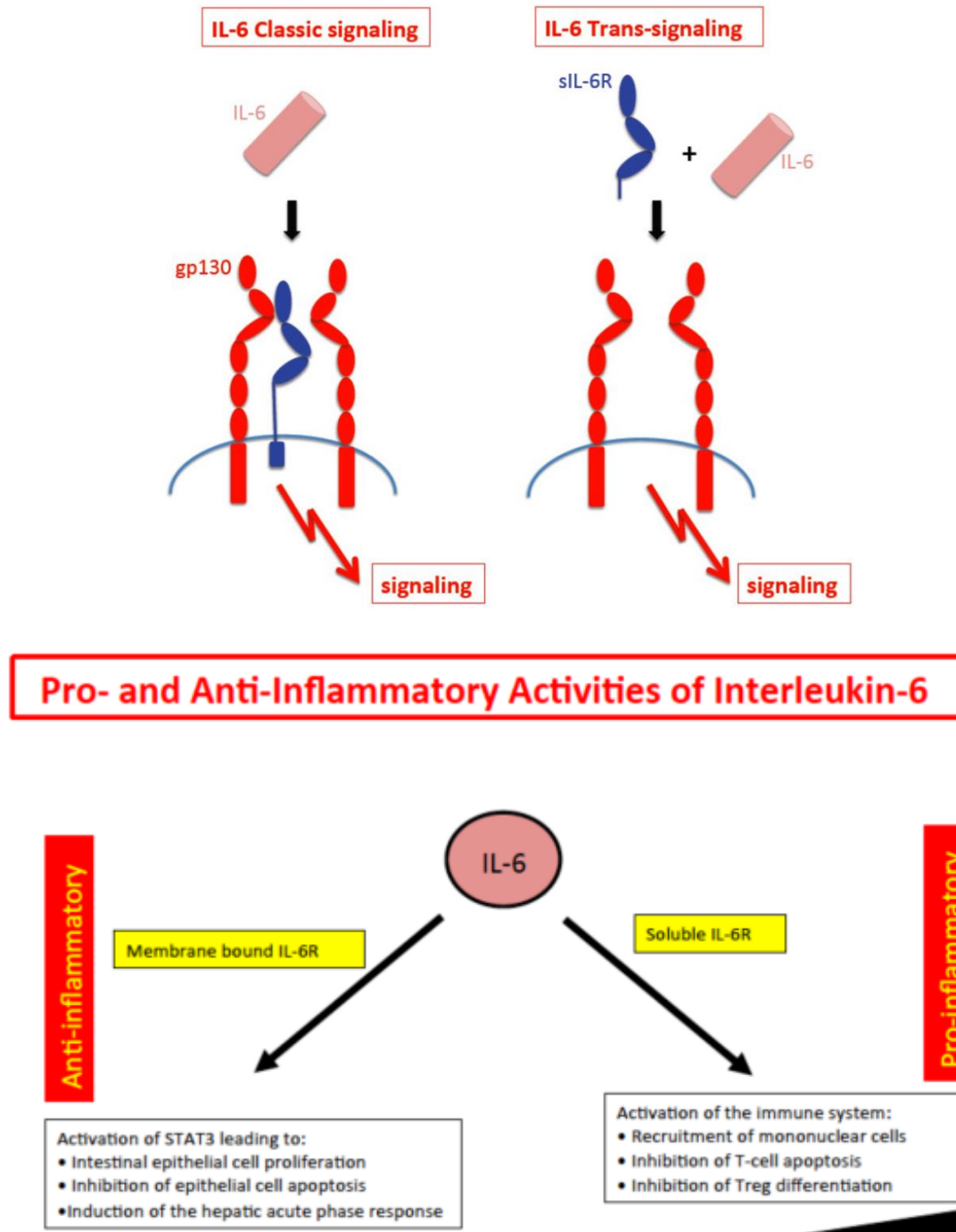


Figure 10: IL-6 signaling pathways (A-above) and IL-6 pro- and anti-inflammatory activities (B-below) (15).

A soluble extracellular form of the IL-6 receptor can replace the receptor and induce signaling by what is called “trans-signaling”. Studies have shown that IL-6 trans-signaling is pro-inflammatory while classic IL-6 signaling is needed for regenerative or anti-inflammatory activities such as proliferation of intestinal epithelial cells, inhibition of epithelial apoptosis and activation of the hepatic acute phase response (figure 10 B) (15).

3.3 Anti-inflammatory cytokines

Anti-inflammatory cytokines play a central role in the inflammation response as they are involved in the return to immune homeostasis and therefore are responsible in preventing the development of chronic inflammation.

3.3.1 Apolipoprotein A-1 (APOA-1) :

APOA-1 is a multifunctional protein and the major component of high-density lipoproteins (HDL) produced primarily by the liver and to a lesser extent by the intestine. APOA-I is involved in the trafficking of cholesterol as well as the regulation of inflammatory and immune response via anti-inflammatory effects.

As mentioned before, modified LDL can act as a ligand for TLR2 receptors and trigger pro-inflammatory signaling. On one side, high concentrations of modified LDL will induce cholesterol influx and accumulation inside macrophages, on the other side, APOA-1 induces cholesterol efflux and therefore has anti-inflammatory properties (11,16). Indeed, lipid poor APOA1 molecules produced by the liver, can pass through the artery wall to interact with ATP binding cassettes (ABC transporters) present on macrophages. These transporters promote the efflux of cholesterol onto lipid poor APOA1 molecules loading them into HDL molecules (figure 11).

In addition, it has been demonstrated that acute inflammation following LPS injection in mice caused an increase in micro-RNA that in turn reduced ABC transporter expression, therefore reducing cholesterol efflux. These results highlighted the importance of APOA-1 in cholesterol efflux from macrophages. Because it is opposed to the cholesterol influx as well as TLR2 activation, APOA-1 is regarded as an anti-inflammatory marker.

There is also evidence that, in humans, HDL can bind to LPS, a Toll Like Receptor activator. In fact, lipoproteins carry the LPS-binding protein that can bind to LPS and act as a cofactor to neutralize LPS (17). Therefore, high levels of APOA-1 would partially inhibit TLR signaling in monocytes and macrophages and decrease pro-inflammatory responses (11).

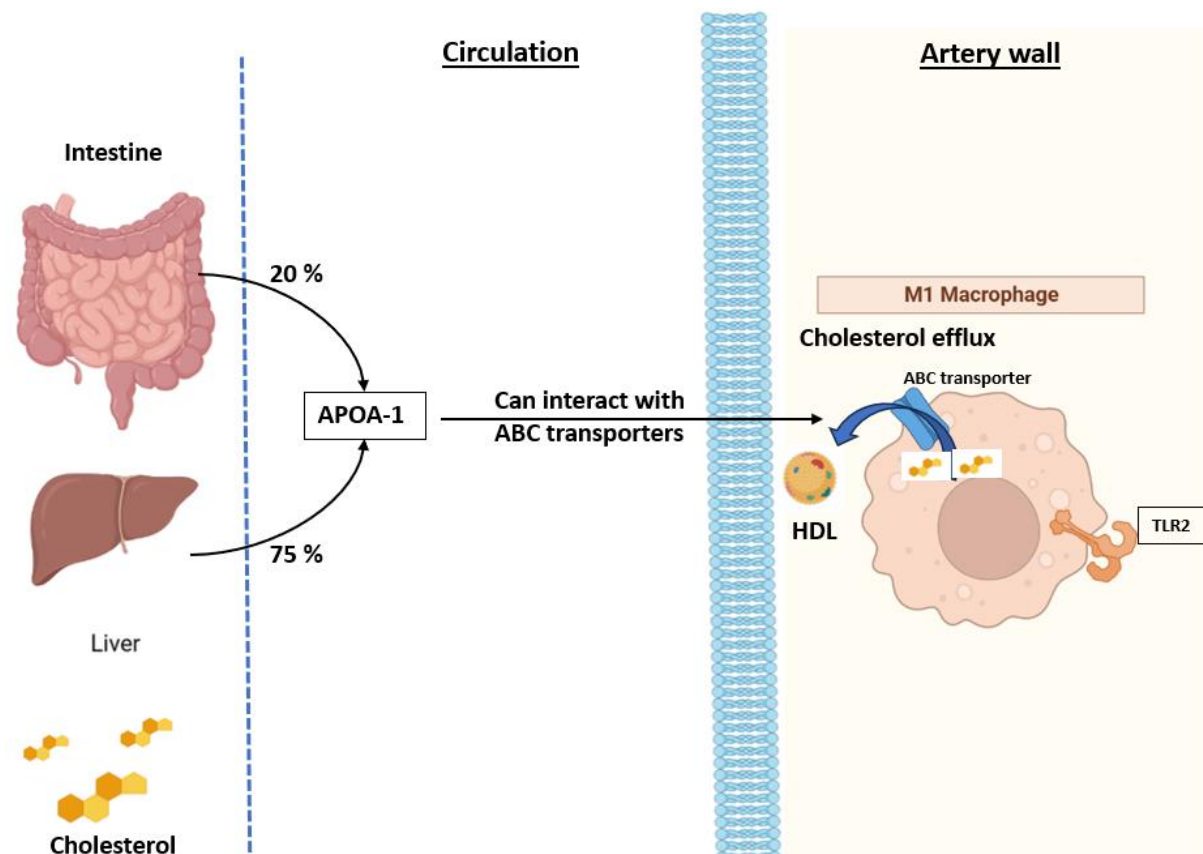


Figure 11: APOA-1 implication in cholesterol homeostasis. Modified from Tall et al., (2015).

3.3.2 Interleukin 10 (IL-10) :

Interleukin 10 belongs to the anti-inflammatory cytokine group along with IL-4, IL-6, TGF- β , IL-37. IL-10 is key to the anti-inflammatory response as it is produced by almost all immune cells associated with both innate and adaptive immunity (8). Its primary effect is to suppress the actions of pro-inflammatory cytokines such as IL-6, TNF- α and IL-8 (5,8). In this role, members of the IL-10 family exert a host defense mechanism that is essential for the maintenance of epithelial layer integrity and tissue healing.

Binding of IL-10 dimers to their tetramer receptor consisting of two IL-10R α (IL-10R1) and two IL-10R β (IL-10R2) chains activates tyrosine kinase 2 (Tyk2) and Janus tyrosine kinase 1 (JAK1), which phosphorylate IL-10R α (figure 12). STAT3 binds to phosphorylated IL-10R α , then STAT3 is phosphorylated by JAK1. Finally phosphorylated STAT3 dimerizes then translocates to the nucleus where it binds to STAT-binding elements within the promoters of various IL-10 target genes, such as suppressor of cytokine signaling 3 (SOCS3) gene. SOCS3 induction has been found to correlate with decreased expression of the pro-inflammatory cytokines TNF- α and IL-1 β (18).

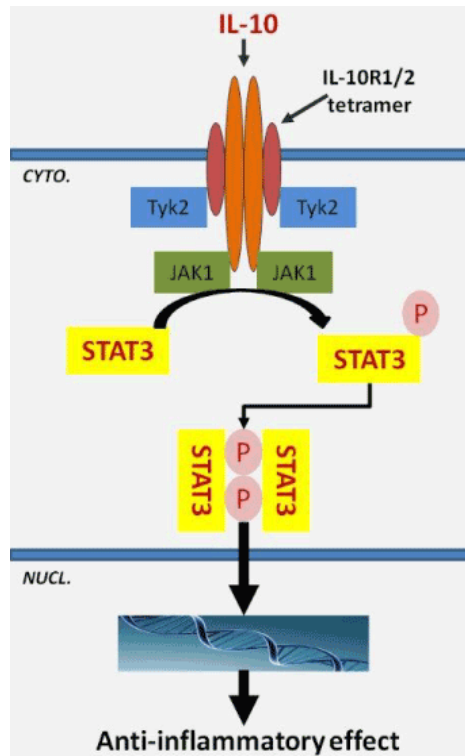


Figure 12: IL-10 signaling pathway.

3.3.3 Sirtuin 1 (SIRT-1) :

Sirtuin-1 is the most evolutionarily conserved mammalian member of the Silent Information Regulator family encoding a key orthologue of nicotinamide adenine dinucleotide-dependent class III histone deacetylase (19). As such, one of its roles is to deacetylate histones in a NAD⁺ dependent manner. Evidence suggests that SIRT-1 specifically deacetylates components involved in the regulation and transcription of genes relevant for aging such as the NF- κ B transcription factor. Thus Sirt-1 is directly linked to the downregulation of inflammation related genes (20).

Moreover, it was revealed that Sirt-1 acted as an anti-inflammatory and is directly involved in inflammaging. Indeed, loss of Sirt-1 activity following gene knockout in zebrafish resulted in an upregulation of classic pro-inflammatory genes (IL-1 β , IL-6, IL-8, TNF- α), an increase in reactive oxygen species production, apoptosis and a reduced lifespan (19,21).

4 Inflammaging

Inflammaging is a relatively new term derived from inflammation and aging. It is defined as a chronic age dependent inflammation involved in many diseases occurring at an advanced age. Inflammaging implies a dysregulation of the inflammation/immune response that can appear with the progression of advanced age. Typically, in young individuals, short-term acute pro-inflammation has the capacity to be resolved. But if it persists, it can evolve into a low-grade chronic inflammation that negatively impacts innate immune cells and is then referred to as inflammaging. Indeed, continuous infections are managed with production of pro-inflammatory cytokines, and this is positive for early survival against infections. It therefore may become detrimental for an organism with a long life as the balance of those cytokines is dysregulated (22).

Inflammaging appears to be mainly caused by the accumulation of proinflammatory tissue damage. However, there is also evidence that the failure of an increasingly dysfunctional immune system, the propensity of senescent cells to secrete proinflammatory cytokines, the enhanced activation of the NF- κ B transcription factor, or the occurrence of a defective autophagy response are also players (5).

NF- κ B is a protein belonging to a large family of transcription factors that act as major regulators of innate and adaptive immunity and inflammatory responses. They are kept inactive in the cytoplasm through their interaction with inhibitors. A multitude of signals originating from pattern recognition receptors, antigen receptors, TNF and IL-1 cytokine families lead to phosphorylation of the NF- κ B inhibitor which allows for the dissociation from the NF- κ B transcription factor. This in turn allows NF- κ B to migrate to the nucleus and regulate inflammatory gene expression (23).

Initially, inflammation was not considered a standalone hallmark of aging but was rather grouped within “altered intercellular communication”. Indeed, the inflammation response involves a lot of intercellular communication through cytokine production. With recent studies, there is enough evidence to consider chronic inflammation as its own hallmark however it remains at an intersection with other hallmarks such as altered intercellular communication and dysbiosis (figure 1).

5 Liver

5.1 Introduction and liver anatomy

In histology the liver can be represented in three different lobules: the hepatic lobule, the portal lobule, and the liver acinus. Those lobules are defined as small functional divisions of the liver defined at a microscopic scale (figure 13).

The basic structure of the liver is the hepatic lobule with a hexagonal. The centrilobular vein is in the center of the lobule. The portal canal or portal triads consisting of the hepatic artery, the hepatic vein and the bile duct can be found on the extremities, between the lobules.

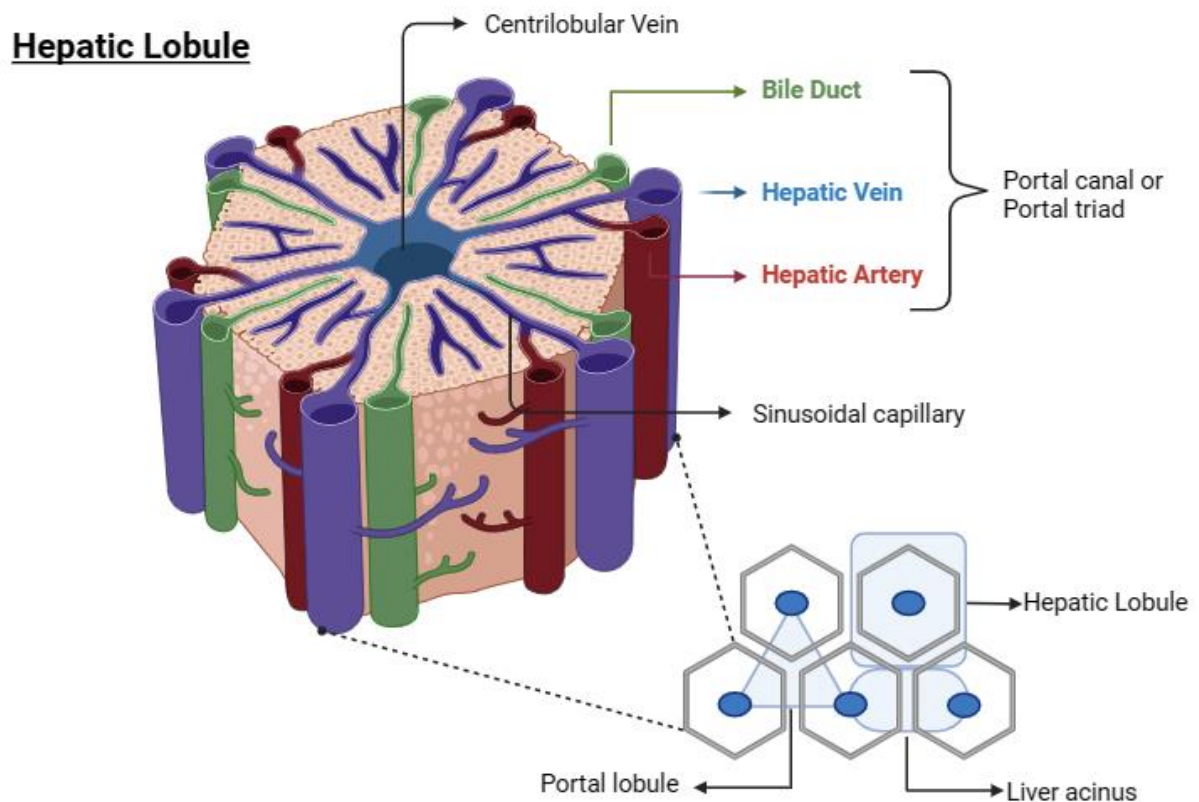


Figure 13: Hepatic Lobule.

Afferent blood enters the liver and the hepatic lobules via the hepatic vein and branches of hepatic arteries in the portal canal, and then travels to the interior of the lobule via the sinusoidal capillaries. Efferent blood exits via the centrilobular veins and travels towards the circulation through the hepatic venous system. The primary cell type of hepatic lobules are hepatocytes: they are arranged against the sinusoidal capillaries via their basal faces and against each other on their apical faces. Apical membranes of bonded hepatocytes form a bile duct. Hepatocytes represent 80% of the liver total mass and are key to all liver functions (20).

The portal lobule is a histological model centered on the bile duct and the bile secretion role of the liver (figure 13 and figure 14). The hepatic liver acinus represents the functional unit of the liver and is therefore oriented around the afferent vascular system (figure 13). The acinus is roughly divided into three zones that correspond to the distance from the arterial blood supply (figure 14).

5.2 Liver multiple roles

The liver is a major metabolic and endocrine organ that is essential for maintaining whole body homeostasis via regulation of energy metabolism, xenobiotic and endobiotic clearance, and molecular biosynthesis.

Main roles are highlighted within figure 14: synthesis and secretion of bile; metabolism of carbohydrates, proteins and lipids; synthesis and regulation of plasma proteins and hormones; storage of specific compounds like vitamins and iron; detoxification of blood and bilirubin clearance; catabolism of many different molecules and red blood cells such as hemoglobin and hormones; and immune functions (24–26).

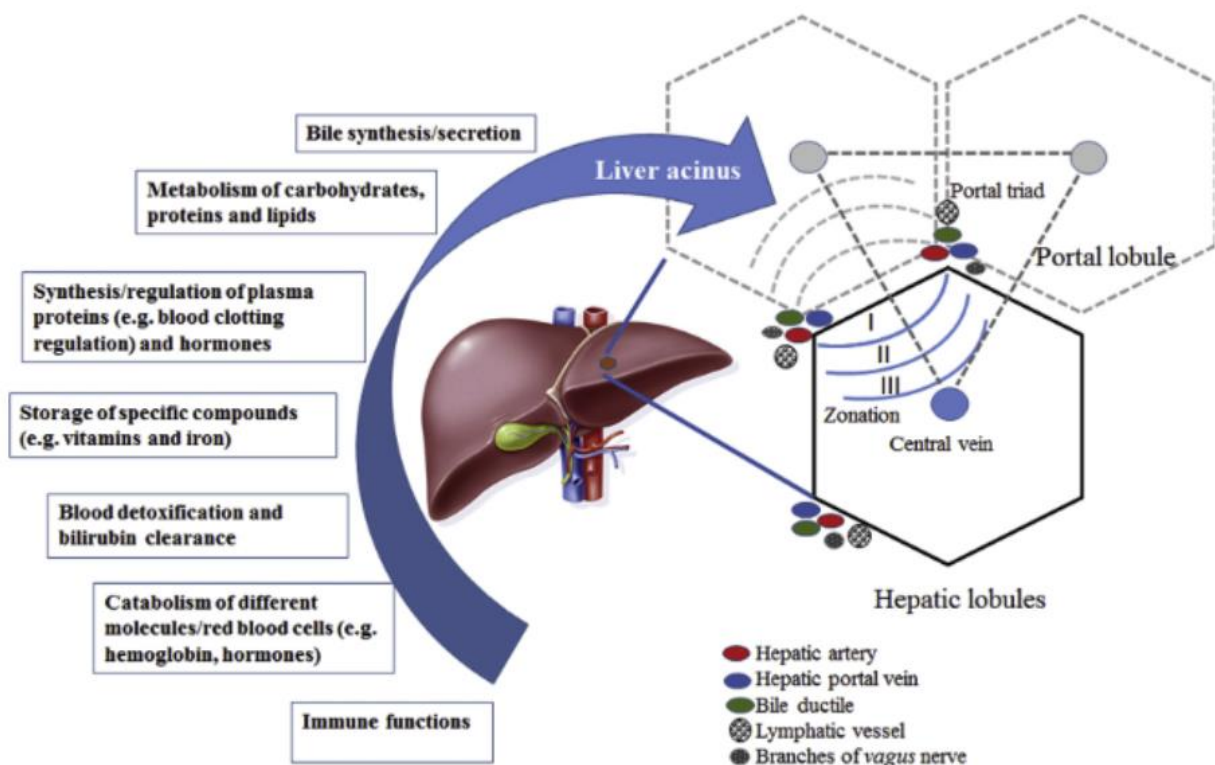


Figure 14: Liver functions from “The peculiar aging of human liver” (25).

The liver has a wide range of functions, therefore any age-related changes impacting liver functions can induce dramatic age-related-diseases. In humans, the liver can be subject to immune, metabolic, neoplastic, developmental, and infectious diseases often resulting in morbidity and mortality. Although, the liver is a unique organ possessing a great capacity of regeneration, this capacity is limited and does not accommodate well with severe acute or chronic injury, like inflammation (24–26).

5.3 Inflammation in liver

Inflammation within the liver can directly induce metabolism issues such as nonalcoholic fatty liver disease (NAFLD) which affects 40% of the Western adult population. NAFLD consists of a large array of diseases from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH) which is the progressive form of the disease and is characterized by inflammation, cellular damage, and fibrosis. It is associated with Type 2 diabetes, obesity, and insulin resistance (24).

Kupffer cells, also known as liver macrophages, have a major role in the innate immune response to liver infections. Kupffer cells are localized within liver sinusoids (see in figure 13) accounting for 30% of sinusoidal cell mass. There are two types of Kupffer cells, M1 and M2 cells. M1 Kupffer cells produce pro-inflammatory mediators while M2 cells produce anti-inflammatory mediators such as IL-10. The inflammation in the liver is regulated through the balance of these two cell types. They function by recognizing and removing pathogens and dangerous molecules via PRRs and are divided into two families: TLRs and NLRs (Nod Like Receptors). As an example, M1 Kupffer cells respond to LPS via their TLR4 receptors to produce many pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-12, IL-18 as well as chemokines (24).

6 *Nothobranchius furzeri* model

For years, zebrafish have been used as aging models as well as yeast, fruit flies, nematodes, and mice, allowing scientists to make major discoveries. On one hand, fruit flies and nematodes have a short lifespan, a useful characteristic for aging studies, but they are not representative of vertebrates. Some aging aspects such as stem cells dynamics or tumorigenesis cannot be studied through these models. On the other hand, common vertebrate models like mice or zebrafish have a longer lifespan which is a limitation for aging studies (27,28).

The ideal scenario would include a vertebrate model with a short lifespan. Studies on these types of models could lead to a better understanding of the functioning of aging mechanisms as well as to explore the wide diversity of aging phenotypes present in nature. *Nothobranchius furzeri* has become an emerging model to study aging.

6.1 Description of the model

Wild *N. furzeri* live in small ponds in Mozambique and Zimbabwe, that are only present during the rainy season. To survive during the dry seasons these fishes have evolved an annual life cycle.

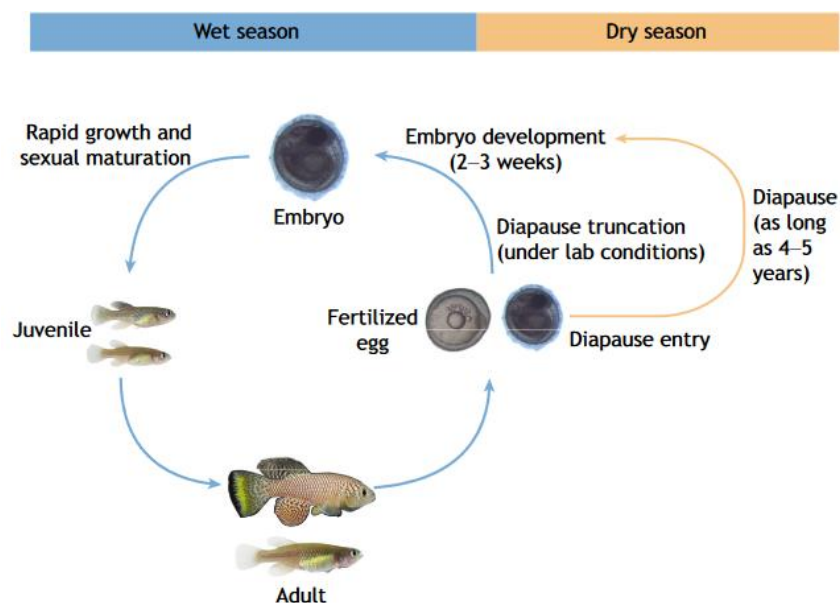


Figure 15: Lifecycle of wild *N. furzeri* (27).

They lay eggs which can enter diapause, a prolonged embryonic state where development can cease up to 4-5 years (figure 15). After hatching during rainy seasons, the larva grows rapidly and quickly reaches sexual maturity (2-3 weeks). With this exceptional lifespan of 4 to 9 months that is conserved in laboratory conditions, this makes *N. furzeri* the vertebrate with the shortest lifespan in captivity (27,28).

Current laboratory aging studies on *N. furzeri* include two strains: the GRZ strain and the MZM strain (figure 16). The GRZ strain is derived from a population collected in 1968 in Zimbabwe. The GRZ strain recorded lifespan is the shortest among all *N. furzeri* species and

ranges from 9 to 16 weeks depending on the breeding conditions. Since the original GRZ strain, multiple other wild strains were collected and derived more recently (29). The MZM strain has a longer lifespan and can reach up to 23 to 28 weeks of median lifespan.

Even though lab conditions have extended GRZ strain lifespan, they still have the shortest lifespan of an animal kept in captivity, in our lab it is 4 to 9 months (16 to 36 weeks).



Figure 16: GRZ and MZM strains of males *N. furzeri* (28).

6.2 Aging in *Nothobranchius furzeri*

N. furzeri shares similar molecular and cellular aging characteristics reported in humans and vertebrate animal models (27). In addition, aging phenotypes like loss of pigmentation (*figure 17*), kyphosis (curved spine), defective vision, locomotion dynamic issues, muscle loss (30,31) and a higher risk of cancer (32), are similar to those observed in humans.

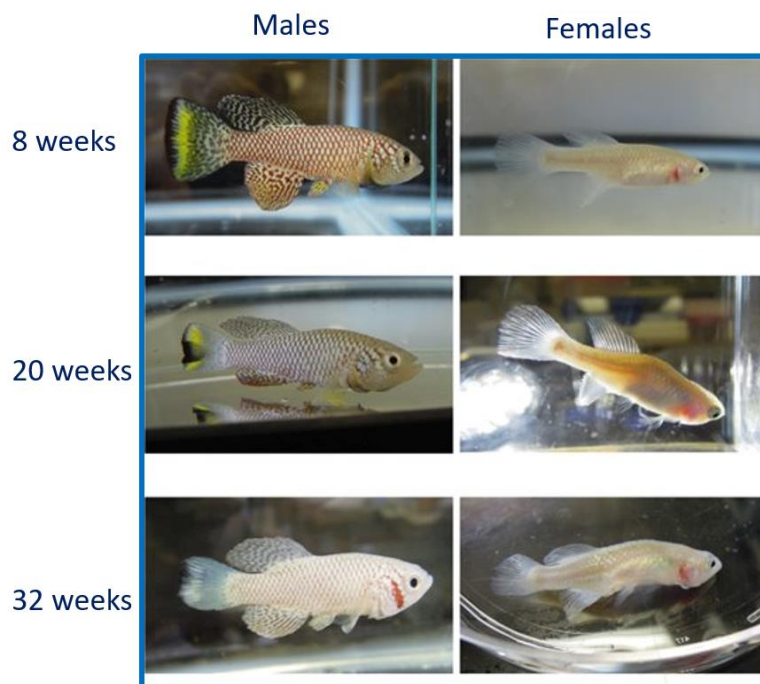


Figure 17: Male and female *N. furzeri* at several ages (GRZ strain) (29).

In this manner, a study revealed the similarity of the immune system between humans and two species of fish (*Danio rerio*; zebrafish and *Nothobranchius furzeri*; African turquoise killifish) (33). Multiple genes involved directly or indirectly to inflammation were found to be upregulated with advanced age in these species within blood, liver and skin. The *marco* gene for example, encodes for a protein found on macrophages and other immune cells, and acts by binding and clearing pathogens during the inflammatory response. Other genes involved in inflammation, like coagulation factor 2 (mediation between coagulation and inflammation), genes coding for membranous receptors (either activating antigen presenting cells or degrading cells surface receptors of leukocytes) were also found to be upregulated with aging (33).

Finally, multiple studies over the last two decades showed that zebrafish is now recognized as good model to study liver development as well as liver linked diseases. Indeed, the rapidly developing methods for gene sequencing in combination with enhanced mutant screening tools allowed identification of liver phenotypes. Such studies were not developed on *N. furzeri* liver, but some evidence showed high degree similarity between Zebrafish and *N. furzeri* genomes (3,24,26,34).

Together these are good indicators that *Nothobranchius furzeri* could be a good aging model, especially in our case to study inflammation within the liver.

Objectives

The aim of this master's thesis was to measure relative gene expression of inflammatory markers (TNF- α , TLR2, IL-8, IL-6, IL1- β , APOA-1, SIRT-1, IL-10) within the liver of the selected model *Nothobranchius furzeri* during aging. We hypothesized that advanced age correlates with two events: the increase in pro-inflammatory gene expression, and the decrease in anti-inflammatory gene expression.

To address these objectives, liver samples of both male and female *N. furzeri* were collected at four different timepoints 6,14,20 and 26 weeks of age. RNA was extracted from the livers to synthesize cDNA. Primers of the genes of interest as well as for selected reference genes (HPRT, GAPDH, EF1 α , TBP, ACTB, INSR) were first optimized for RT-qPCR. Additionally, the most suitable reference genes were selected using geNorm algorithm. Following this, the expression of the genes was measured across the four different timepoints. The data obtained (CT values) was used to plot the relative gene expression of the genes of interest across aging. Statistical analysis was carried out to assess whether the impact of age and the impact of sex was significant.

Material and Methods

1 *Nothobranchius furzeri* breeding

Both male and female *Nothobranchius furzeri* (GRZ strain) were used in the following experiments. Fish were reared and housed within ZebTEC Active Blue standalone aquaria (Techniplast, Italy) at the ANCA platform fish facility (LIBST, UCLouvain). Water was maintained at a temperature of 28°C, a pH between 7 and 7.5, a conductivity between 600 and 700 μ S. All fish were reared under a 12-hour light/dark cycle (lights on 07h00 and lights off at 19h00).

From hatching until 2 weeks of age, larvae were fed *Artemia salina* nauplii (brine shrimp, Great Salt Lake, Utah, USA, Ocean Nutrition, Europe, Essen, Belgium) twice daily, once in the morning and once in the afternoon. Between 2 weeks of age and the onset of sexual maturity (approximately 6 weeks of age) the fish are slowly introduced to *Chironomus* spp. larvae (bloodworms, Ocean Nutrition, Belgium) as an additional food source. At sexual maturity, male *N. furzeri* were either housed alone or with 3 to 4 females. Adult fish were fed with *Chironomus* spp. larvae in the morning and in the afternoon as well as *Artemia* once in the afternoon.

2 *Artemia* culture

Artemia cysts were cultured in hatching cones in the fish facility. *Artemia* were incubated in salt water (3,5g /liter InstantOcean), pH 7-7.5, 28°C, under constant aeration. *Artemia* hatched under these conditions within 48 hours. After this period, *Artemia* were collected using a 0.15 mm sieve and rinsed with reverse osmosis water before being fed to the fish.

3 Experimental Fish

Four male and four female *N. furzeri* at each 6, 14, 20 weeks of age and two males and four females at 26 weeks of age were euthanized by immersion in a 0.2% MS-222 (tricaine methane sulfonate) solution for approximately 10 minutes. Death was confirmed by the lack of movement in response to gentle pricks with the tip of a needle on the tail. The fish were weighed and their length from mouth to tail fin was measured before dissection (See Annex 1 for mass and length values). Liver, brain, heart, and muscle tissue was collected, flash frozen in liquid nitrogen and stored at -80°C until used in further experiments. Livers were used in the present study, whereas other organs were stored for other aging-related experiments. All fish were handled in conformity to the regulations of the Animal Ethics Committee of UCLouvain.

4 RNA extraction and quantification

RNA was extracted from *N. furzeri* livers using the Aurum™ Total RNA Mini Kit (Bio-Rad Laboratories, Belgium). The protocol for the following steps was slightly modified. Livers were individually transferred to 1.5 mL Eppendorf tubes. Due to the size and weight of *N. furzeri* livers, 0.5 mL of TRIzol reagent was added to the tubes. Samples were homogenized using an electrical pestle (Sigma Aldrich, Belgium) and incubated for 5 minutes at room temperature. 0.1 mL of chloroform was added to each tube and shaken vigorously for 15 seconds then centrifuged at 4°C (12 000 g for 15 min). Approximately 250 µL of the aqueous phase was extracted then homogenized with an equal volume of 70% ethanol in a new 2 mL tube. The mixture was transferred to an RNA column placed above a 2 mL capless tube. Each sample was centrifuged for 1 minute at room temperature. The filtrates were discarded, and the columns were washed with 700 µL of a low stringency wash solution. Samples were then centrifuged for 10 seconds, and the filtrates were discarded. A mixture of 5 µL DNase and 75 µL DNase dilution solution was added to each column/sample and incubated for 25 minutes at room temperature. 700 µL of a high stringency wash solution was added, then the columns were centrifuged for 10 seconds at room temperature and the filtrates were discarded. Another 2 minute-round of centrifugation was made and the filtrates were again discarded. The columns were transferred to a clean 1.5 mL Eppendorf tube. Finally, 40 µL of elution solution was added to the columns and incubated for 1 minute before centrifuging for 2 minutes at room temperature. 3 µL from each sample was used to determine RNA concentration and purity (Annex 2) using a spectrophotometer (Nanodrop, Thermo Fischer Scientific, Belgium). The remaining RNA extracted samples were stored at -80°C.

5 cDNA synthesis

1000 ng of extracted RNA was used to synthesize cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad laboratories, Belgium). 15 µL of the RNA was added to 0.2 mL PCR tubes on an ice plate. A mixture containing 4 µL of Reaction Mix and 1 µL of Reverse Transcriptase was added to each sample. A control “no RT” tube was made using 1 µL of nuclease free water instead of the RT enzyme. RNA samples were placed in a thermal cycler (Applied Biosystems SimpliAmp Thermal Cycler, Thermo Fisher Scientific, Belgium) where cDNA was synthesized following the temperature and time parameters outlined below. Samples were stored at -20°C.

Table 1: synthesis of cDNA, steps and parameters

Steps	Temperature (°C)	Time (min)
Priming	25	5
Reverse Transcription	46	20
RT inactivation	95	1
Hold	4	/

6 RT-qPCR

6.1 General information

The Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed using 96-well plates (Applied Biosystems, Thermo Fisher Scientific, Belgium) adapted for the qPCR machine used in the AMCB laboratory (StepOnePlus Real-Time PCR System, Applied Biosystems, Belgium). Samples were analyzed in triplicate. In addition, primer optimization included a wide range of dilutions to find the most adapted one to be used. Two negative controls were used; “no RT” and “no cDNA”, which are controls that do not include, respectively, the reverse transcriptase enzyme or cDNA. The GoTAQ® qPCR Master Mix Kit (Promega, Belgium) was used. SYBR reagent was prepared by adding 20 μ L CXR (GoTAQ). The Master Mix is specific to one gene tested and was prepared by adding 10 μ L of the SYBR reagent, 6 μ L of nuclease free water and varying concentrations of forward/reverse primers (according to the desired ratio) for a total volume of 2 μ L. To each well, 2 μ L of cDNA was added followed by 18 μ L of Master Mix. The 96-well plate was sealed and centrifuged at 1200 rpm for 3 minutes.

Finally, the plate was placed in the qPCR machine (Table 2 shows the parameters used. Data was collected using the StepOne™ Software v2.3.

Table 2: Stages and parameters for the PCR run

Stage	Temperature (°C)	Time (min)
Holding stage (1 cycle)	95	02:00
Cycling stage (40 cycles)	95	00:15
	60	01:00
Melt curve stage (1 cycle)	95	00:15
	60	01:00

6.2 Primer selection and design

Primer sequences for the genes of interest were either taken directly from the literature (35) or designed by a previous master student. Primer sequences for the reference genes were previously designed and used in the BNTE laboratory (section 6.d).

Sirt-1 primer sequence was obtained using the NFINgb database. The primer sequence was selected using Oligoevaluator depending on the following parameters: GC content around 50%; primers between 18-30 nucleotides; melting temperature between 60-65°C and maximum difference of 1°C between the forward and reverse primer; amplicon formed between 120-350 nucleotides; an absence of primer dimers; an absence of or the presence of weak secondary structure; GC at the 3' end.

The primers were ordered from Eurogentec, Belgium. Modifications on some primer pairs coming from the literature had to be made since melting temperature discrepancies were observed using the Oligoevaluator program.

Table 3: Primer Information

Protein	Sequence	Length (bp)	Secondary structure	Primer dimer	GC (%)	Melting Temperature (°C)
TLR2 (F)	GCTCAACAACCTGTACAGCTGC	22	Weak	No	54.55	66
TLR2 (R)	GATTGAAGTCTCAGGGTACGACACAT	26	Weak	No	46.15	67.1
APOA-1 (F)	CATTCCAGGAACATAAGGCG	20	Very Weak	No	50	64.2
APOA-1 (R)	GCGTTGTGTTTGTCACTGTACTCT	24	Weak	No	45.83	64.3
IL-6 like (F*) LOC107383629	GGAGGAATTTCAAGGGAACATA	22	Very Weak	No	40.91	62.8
IL-6 like (R*) LOC107383629	CCTCAGGAGAACCATGTAGA	20	None	No	50	59.3
IL-8 like (F) LOC107382165	ATTCATAAAAAACAACAGACAAAAGATCT	28	Very Weak	No	25	62
IL-8 like (R*) LOC107382165	ATCGTATTCACCATCATGTCTC	22	None	No	40.91	60.3
IL-10 (F*)	CCTTACAGCATCATGTCTCTC	21	Very Weak	No	47.2	58.9
IL-10 (R)	TGTCAAAGTCATCGTTTGCT	20	Weak	No	40	60.9
IL-1 β (F*)	CCGACAGCAAGAAACGAA	18	None	No	50	62.7
IL-1 β (R)	TTGTCTCCTTTTTTGTGGCA	20	None	No	40	63.2
TNF- α like (F) LOC107396784	ATTAGAGCATGATGGGATACG	21	None	No	42.86	60.4
TNF- α like (R) LOC107396784	TGTGATGAAACAACCTGCTTCT	21	Very Weak	No	38.1	60
SIRT-1 (F)	ATCAGCAGGAGTCCAATAAGC	21	None	No	47.62	62.1
SIRT-1 (R)	GCTTTTTCAGAAGAACTGTTCGAT	23	Weak	No	39.13	62.5
HPRT (F)	TGAGATCAGAGTGATCGGT	19	Moderate	No	47.4	58.2
HPRT (R)	GCTACTTTAACCATTTTGGGAT	22	Weak	No	36.4	59.8
ACTB-1 (F)	CCTTCCTTCCTCGGTATGG	19	None	No	57.9	63.4
ACTB-1 (R)	AGGTGGGGCAATGATCTTAATC	22	Very Weak	No	45.5	64.9
TBP (F)	CGGTTGGAGGGTTTAGTCCT	20	Weak	No	55	64
TBP (R)	GCAAGACGATTCTGGGTTTG	20	Very Weak	No	50	64.5
GAPDH (F)	AAAGTCGGAATCAATGGATTTGG	23	Weak	No	39.1	66.6
GAPDH (R)	TTAGTCGGGTCCCTCTCGTG	20	None	No	60	66.4
EF1 α (F)	TTACAGCCAGAAACGCTACG	20	Weak	No	50	62.9
EF1 α (R)	TTAAACCAGCCCATCTTGTAC	22	None	No	45.5	64.9
INSR (F)	TGCCTCTTCAAACCCTGAGT	20	Weak	No	50	63.8
INSR (R)	AGGATGGCGATCTTATCACG	20	Very Weak	No	50	63.9

In blue: Genes of interest – In orange: reference genes. Abbreviations: TLR2; toll-like receptor 2, APOA-1; apolipoprotein A-1, IL; interleukin, TNF- α ; tumor necrosis factor-alpha, SIRT-1; Sirtuin or silent mating-type information regulation 1, HPRT; Hypoxanthine-guanine

phosphoribosyltransferase, ACTB-1; Actin, cytoplasmic 1, TBP; TATA-binding protein, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, EF1 α ; Elongation factor 1- alpha, INSR; Insulin receptor. F; forward, and R; reverse. Primers marked () were published from Vanhunsel et al., 2021.*

6.3 Primer optimization

Before measuring gene expression across several ages in *N. furzeri* liver extracts, primer parameters were optimized. The goal is to obtain a primer efficiency of 100%, which indicates that the primer pairs can double the amount of DNA after each cycle. The primer optimization step is essential to determine that the cDNA is amplified adequately (36). To calculate primer efficiency five two-fold dilutions were made with the cDNA. Each dilution was measured in triplicate and the mean CT values were plotted on the y-axis with a logarithmic scale of the dilutions on the x-axis. Using the slope of the standard curve, the primer efficiency was calculated:

$$\text{Efficiency (\%)} = \left(\left(10^{\frac{-1}{\text{slope}}} \right) - 1 \right) * 100$$

Primers, in which an efficiency between 80-120 % was calculated were accepted to use in future experiments. We also set 0.95 as a cut off for the slope of the standard curve for concentration dilutions. Three primer ratios (forward/reverse) were also tested; 500:500, 500:250 and 250:500.

6.4 Reference genes

Reference genes are a baseline for gene expression. In this context, their main role is to remove the technical gene expression variation (appearing while measuring genes of interest expression) as much as possible to end up with the true biological changes (37). In other words, the expression of the genes of interest must be standardized the reference genes to be meaningful. For this purpose, reference genes are chosen as they are believed to be constantly expressed across the different ages. For this study we selected ACTB1, TBP, GAPDH, HPRT, EF1 α and INSR, which were previously designed in the lab.

To test for significance differences, gene expression across aging was normalized to the geometric mean of gene expression of the reference genes. Typically three to five reference genes are suggested for accurate normalization (37). In addition to testing primer efficiency of the reference genes, we also tested their stability using the GeNorm algorithm of ReFinder. ReFinder is a user-friendly web-based comprehensive tool developed for evaluating and screening reference genes and GeNorm is an algorithm used to determine the most stable reference genes from a set of tested candidate reference genes in a given sample (38). Among the six references genes tested (ACTB, TBP, EF1 α , GAPDH, INSR and HPRT), the three most stable were selected for further analysis: HPRT, TBP and EF1 α (figure 18).

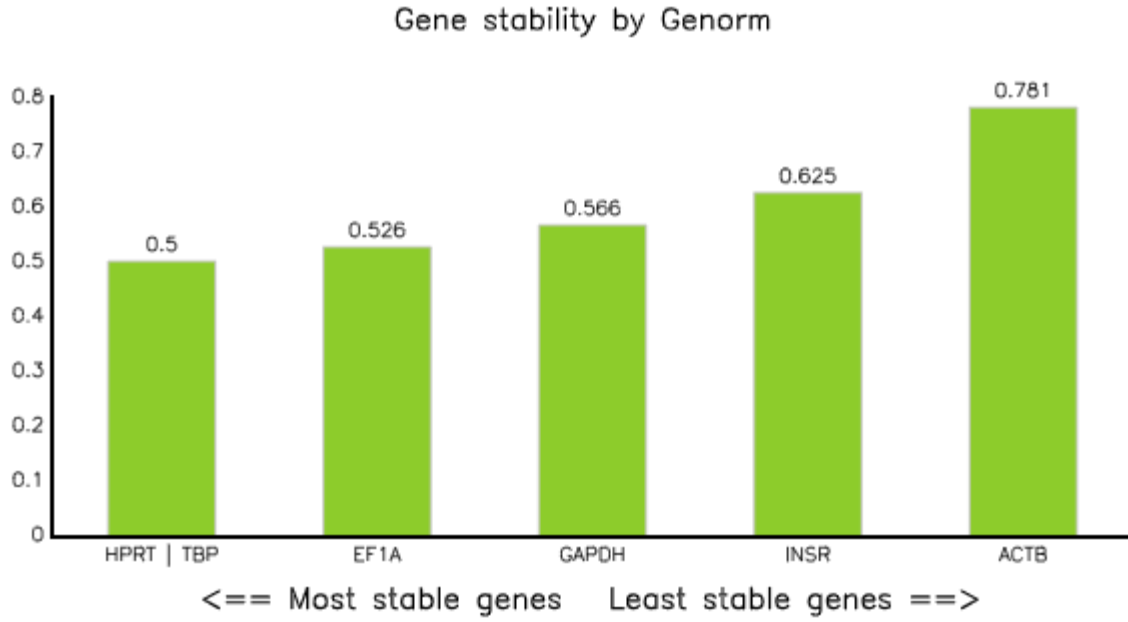


Figure 18: Reference gene stability using the GeNorm algorithm.

6.5 Gene expression across ages

Expression of the genes of interest was measured in triplicates for each timepoint: 6, 14, 20 and 26 weeks of age. Each timepoint included eight samples; four males and four females except for the 26-week group which included two males and four females.

Triplicates were used to assure reproducibility: CT means were calculated with the geometrical mean of the three CT values; a standard deviation was also calculated based on those three values. Based on the results obtained after primer optimization, primers of genes of interest were 2x diluted while primers of reference genes were 10x diluted.

96 wells plate were used for this purpose. One plate was used to test one gene. An example of plate design can be seen in annex 3. Each sample was placed in triplicate and five controls were realized: one for each group of age, and one with no reverse transcriptase enzyme (for the 14 weeks group of age in annex 3 – control for other groups of age were tested on another plate). Disposition of samples on the plate were mixed between each plate to avoid errors linked to the plate.

6.6 Data treatment and statistical analysis

To answer this thesis objective, we first needed to represent relative gene expression (RGE) of the genes of interest across our 4 aging timepoints (6, 14, 20, 26 weeks). To do so, a CT mean of our triplicates CT values was calculated for our genes of interest (goi) and for our 3 selected reference genes (ref). The difference between their means is called ΔCT :

$$\Delta CT = (CTmean_{goi}) - (CTmean_{ref})$$

Relative quantification (RQ) is described using the following formula:

$$RQ = 2^{-\Delta CT}$$

Relative quantification helped us representing relative gene expression (RGE) by plotting

$$RGE = \frac{RQ}{Mean RQ_{6weeks}}$$

on the y-axis across aging on the x-axis. *Mean RQ_{6weeks}* represents the mean RQ of all samples of 6 weeks of age, for every gene tested. This way, on RGE graphs, values of 14,20- or 26-weeks group of age are always expressed relatively to the 6 weeks group of age.

Statistical analysis was done using GraphPad Prism and Rstudio softwares. First, we needed to ensure that our data (RGE or RQ) was normally distributed meaning data follow a gaussian tendency on their domain. Therefore, before any analysis a *Normality* and *LogNormality* test were made for every gene. The results indicate that our experimental design model should be transformed in a logarithmic scale for our data to be normally distributed.

We then conducted statistical analysis following 2 hypotheses:

- 1) There is an effect of age on the gene expression
- 2) There is an effect of sex on the gene expression

6.6.1 Impact of age on gene expression

For this analysis, the ordinary one-way ANOVA test was made assuming gaussian distribution of LOG data. A p-value under 0.05 would confirm that age has indeed an effect on the gene expression. In addition, a multiple comparison test was performed (using Tukey test) to determine any significant differences between 2 groups of age. Other tests were possible for this, such as the Bonferroni test and the results obtained could be different. Finally, two conditions of applications of this ANOVA test were performed by checking both the normal distribution of the residues as well as the heteroscedasticity of the residues.

6.6.2 Impact of sex on gene expression

For this analysis, an unpaired t-test with welch correction was performed to detect any significant difference between all males and all females' samples. This was made assuming no homogenous residues between males and females. As a reminder p-value under 0.05 would reveal a significant difference.

Results and discussion

1 Results

1.1 Primer optimization

To measure gene expression of our genes of interest across several ages, we first optimized their primers. cDNA synthesized from our RNA samples were used for amplification. Parameters to optimize are the ratio between forward (F) and reverse (R) primers, RNA quantity used for cDNA synthesis and dilutions used to calculate primer efficiency. Multiple primer ratios (F/R) were tested (500/500 – 500/250 – 250/500), 600 ng and 1000 ng were tested for RNA quantity, and finally different dilutions were tested ranging from two-fold to ten-fold. As explained in the *Material and Methods* section, a primer efficiency between 80-120% was considered acceptable.

The standard curves obtained from plotting CT values with the dilutions were used to calculate the primer efficiency and are regrouped in figure 19 (for genes of interest) and figure 20 (for reference genes).

1.1.1 Genes of Interest (GOI)

Out of the eight genes of interest tested, four primer pairs were optimized successfully (efficiency between 80-120%): IL-10, TLR2, APOA-1, IL-8. Following many manipulations, primer pairs for TNF- α , IL1- β and IL-6 could not be optimized for this thesis (i.e., efficiency outside of 80-120%). Primer pairs for SIRT-1 did not go through an optimization process due to time constraint. A summary of optimized parameters for all genes tested can be found in annex 4.

Some of the graphs and their standard curve are made with four dilutions instead of five. This is because in certain cases the highest dilution was not detected during amplification and as a result could not be represented on the graphs.

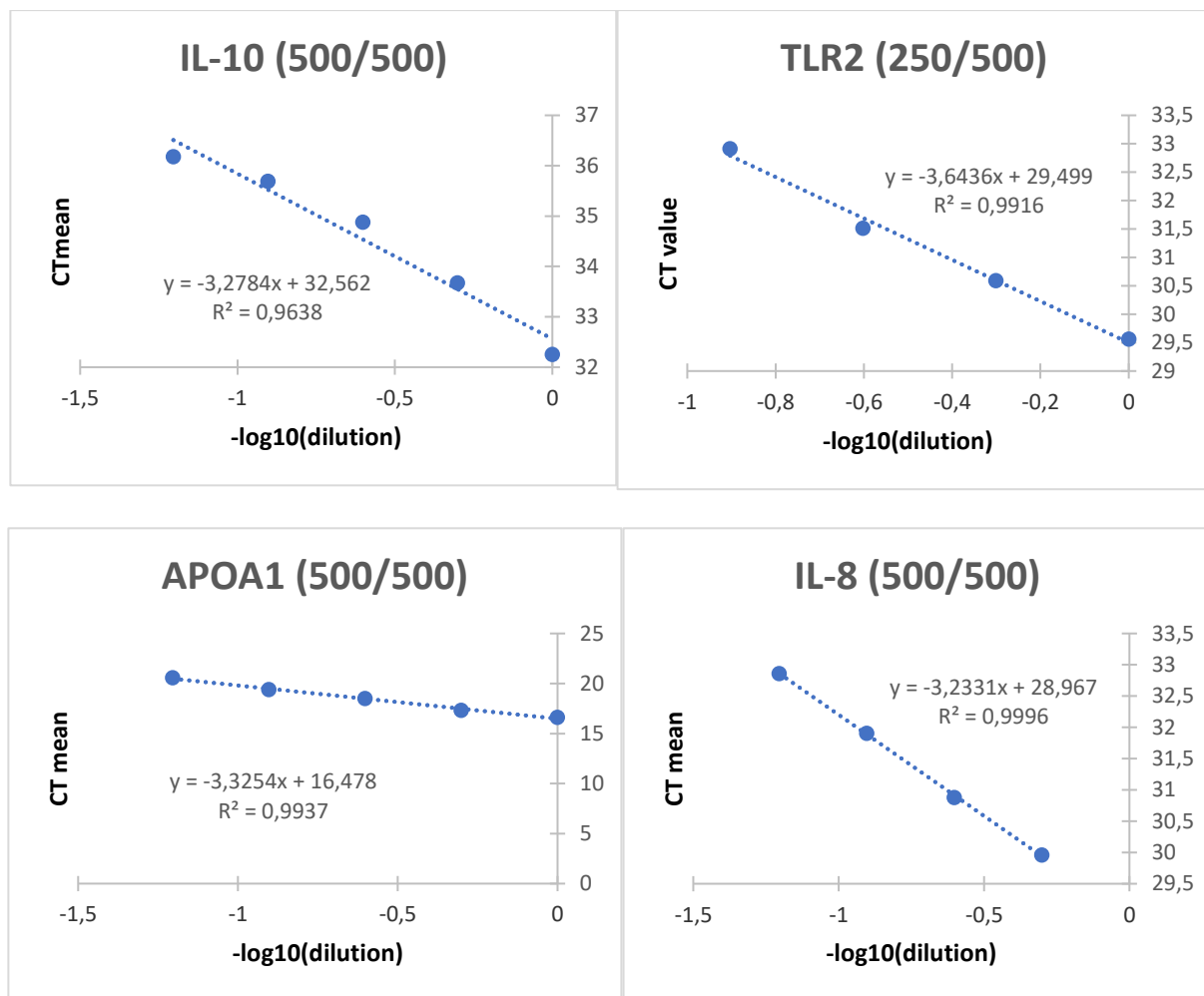


Figure 19: IL-10, TLR2, APOA-1 and IL-8 (genes of interest) primer pair ratio standard curves of the mean CT values according to the $-\log_{10}$ of cDNA dilution of liver samples.

1.1.2 Reference genes

The same optimization process was conducted for the six reference genes: HPRT, TBP, ACTB, GAPDH, INSR and EF1 α (figure 20). A summary of optimized parameters for all genes tested can be found in annex 4.

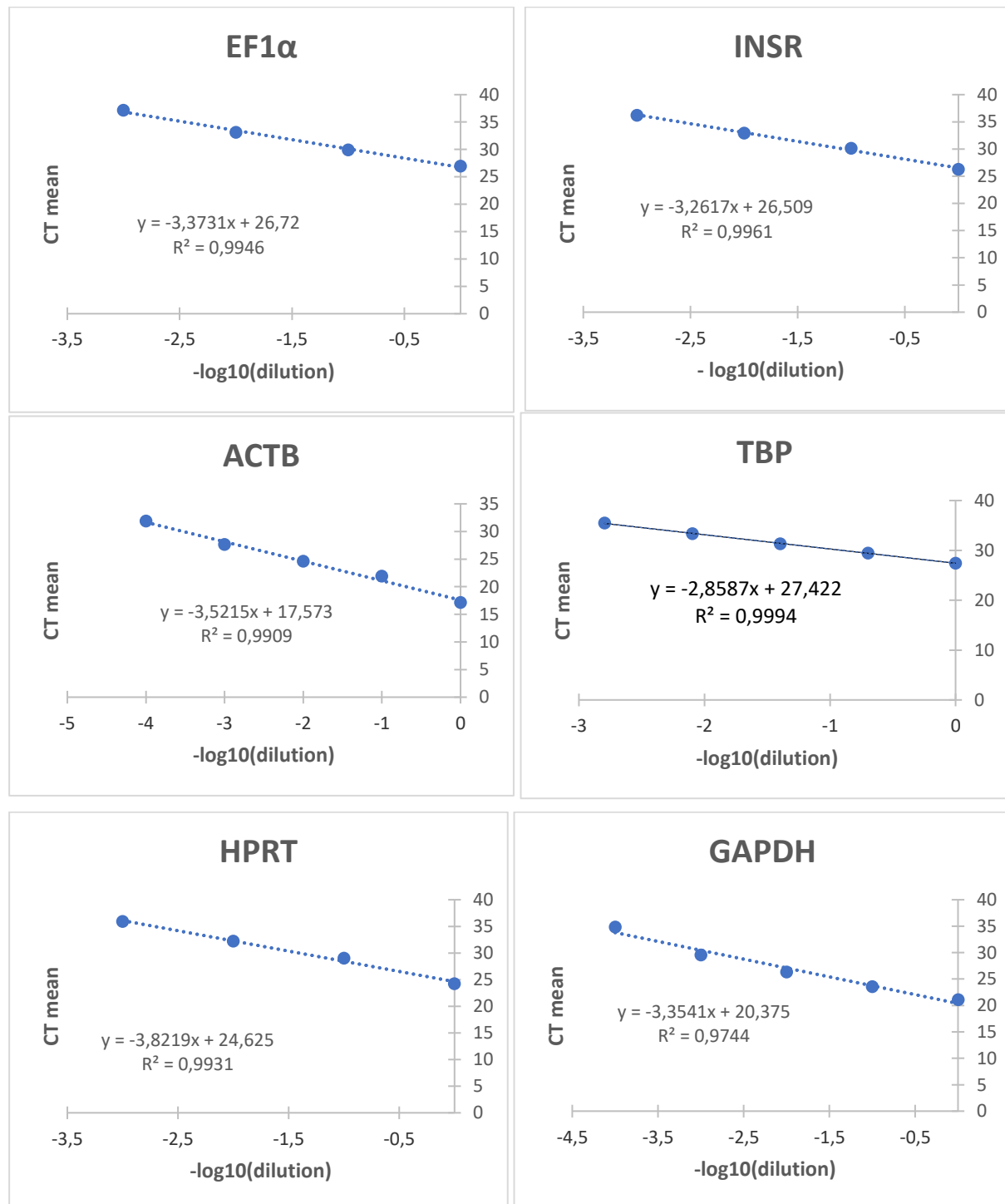


Figure 20: EF1 α , INSR, ACTB, TBP, HPRT and GAPDH (reference genes) primer pair ratio standard curves of the mean CT values according to the $-\log_{10}$ of cDNA dilution of liver samples.

Although the primer efficiency for TBP was slightly higher than 120% (annex 4), it was accepted to be used since the amplification plot and efficiency standard curve are similar to those which passed the efficiency test (annex 4).

1.1.3 Reference genes selection

As mentioned in the *Material and Methods* section, the most stable reference genes were selected using geNorm algorithm. Ct mean values of the expression of the six reference genes were used as input data for geNorm.

Table 4: CT mean values for the reference genes across ages

Age (weeks)	ACTB	EF1 α	GAPDH	HPRT	INSR	TBP
6	22,99	30,45	24,14	24,57	30,62	29,97
14	24,62	31,56	24,39	25,14	30,15	31,29
20	21,62	30,01	24,24	25,13	29,25	30,52
26	23,93	30,66	24,57	25,15	30,45	30,42

HPRT, TBP and EF1 α were the genes that were expressed at the most constant levels across the four age timepoints and therefore were selected for further analysis.

1.2 Gene expression across ages

RT-qPCR was carried out on 30 livers samples: 4 males and 4 females in each age group (6, 14, 20 weeks) except in the 26 weeks group where there were only 2 males and 4 females. For each sample, all genes of interest that passed primer optimization (IL-10, TLR2, APOA-1, IL-8), were tested, as well as for the 6 reference genes selected (HPRT, TBP, ACTB, GAPDH, INSR and EF1 α). After which geNorm was used to calculate the three most stable reference genes. These three reference genes were then used to normalize the expression of the genes of interest.

1.2.1 Relative gene expression Interleukin-10 (IL-10)

The expression of the anti-inflammatory IL-10 was significantly elevated (p -value = 0.0096) at 14, 20 and 26 weeks compared to 6 weeks of age (figure 21). There is no evidence that gene expression differs between the three older timepoints, however it is important to note the high level of variability within these timepoints, which complicate interpretations.

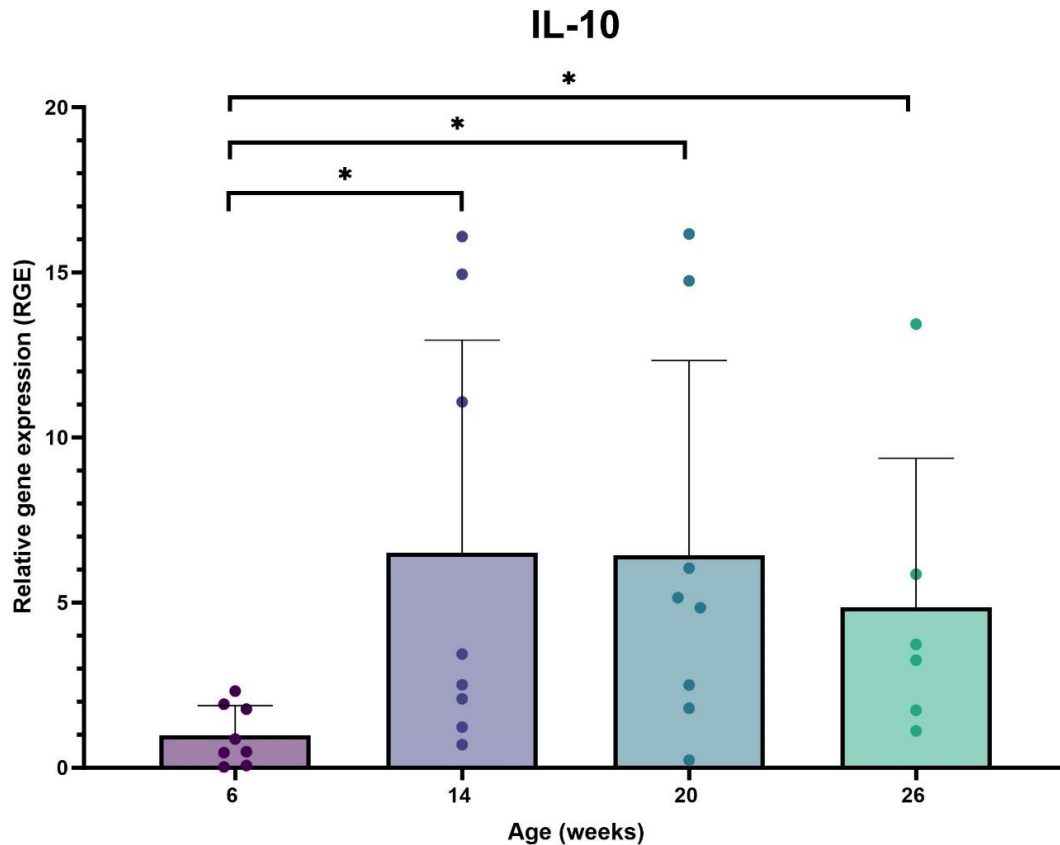
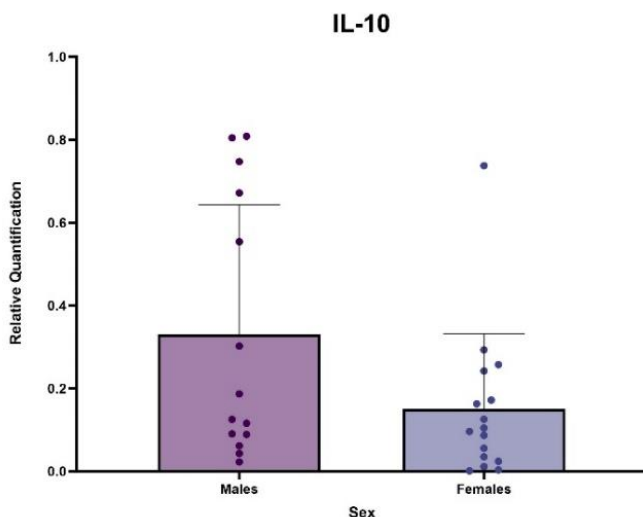


Figure 21: IL-10 relative expression across ages. Data includes both males and females. Each dot corresponds to a sample. $N=8$ (6, 14 and 20 weeks), $N=6$ (26 weeks); $n=3$. (*) indicates a p -value < 0.05.



The results revealed no significant impact of sex on the gene expression of IL-10, however the p -value is close to 0.05 (figure 22).

Figure 22: IL-10 relative quantification of males compared to females. Each dot corresponds to a sample. $N=14$ (males), $N=16$ (females); $n=3$.

Apolipoprotein A1 (APOA-1)

In contrast to IL-10, the expression of the anti-inflammatory APOA-1 did not differ with age. Expression is higher at 14 weeks compared to the other ages; however, significance is not reached due to the variability at this time point (figure 23).

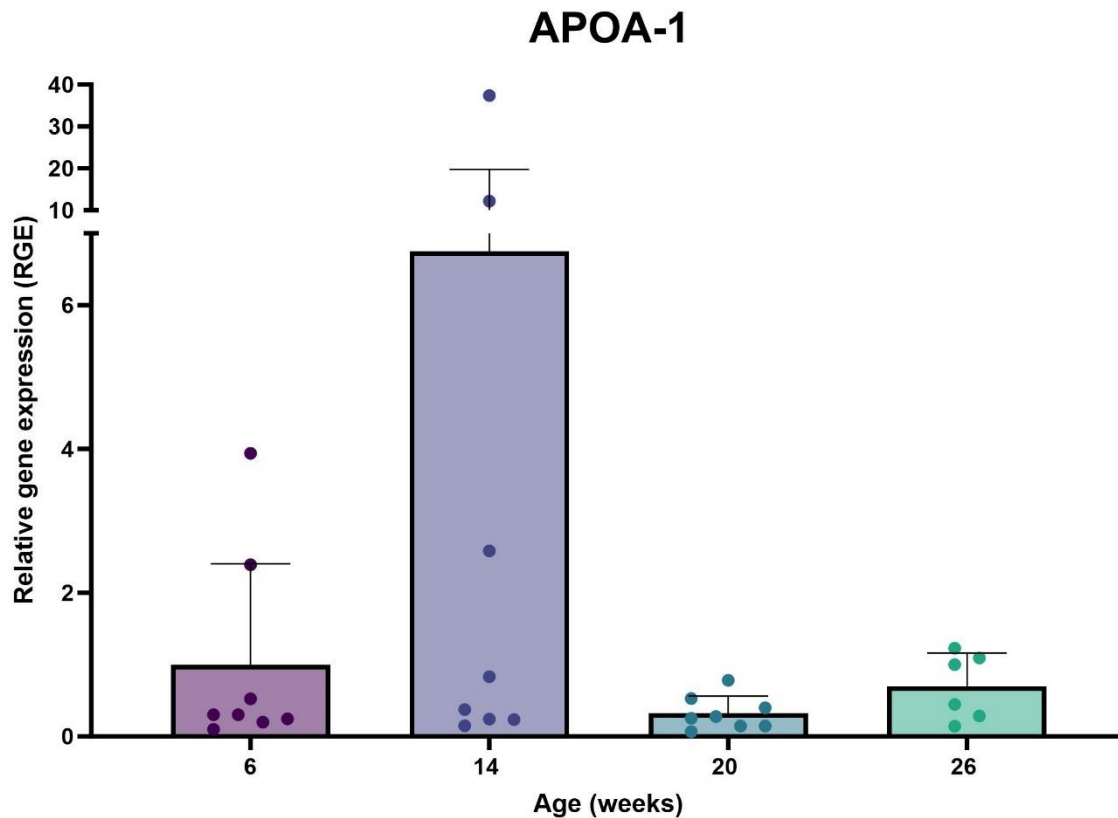
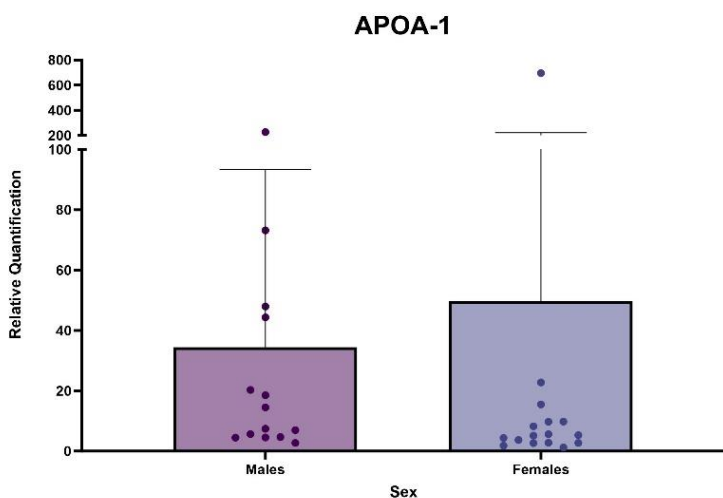


Figure 23: APOA-1 relative expression across ages. Data includes both males and females. Each dot corresponds to a sample. N=8 (6, 14 and 20 weeks), N=6 (26 weeks); n=3.



The results revealed no significant impact of sex on the gene expression of APOA-1 (figure 24).

Figure 24: APOA-1 relative quantification of males compared to females. Each dot corresponds to a sample. N=14 (males) and N=16 (females); n=3.

Interleukin-8 (IL-8)

The expression of the pro-inflammatory IL-8 did not differ with age. Expression is higher at 14 weeks compared to the other ages; however, significance is not reached due to the variability at this time point (figure 25).

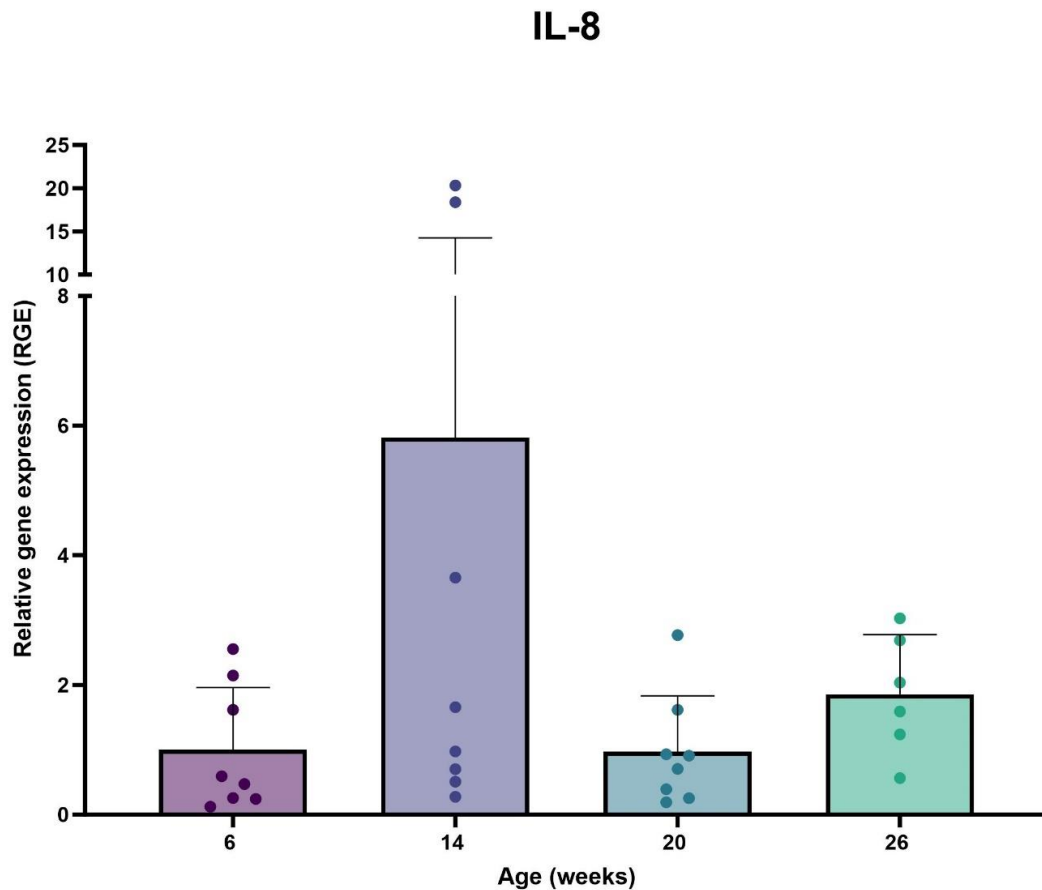
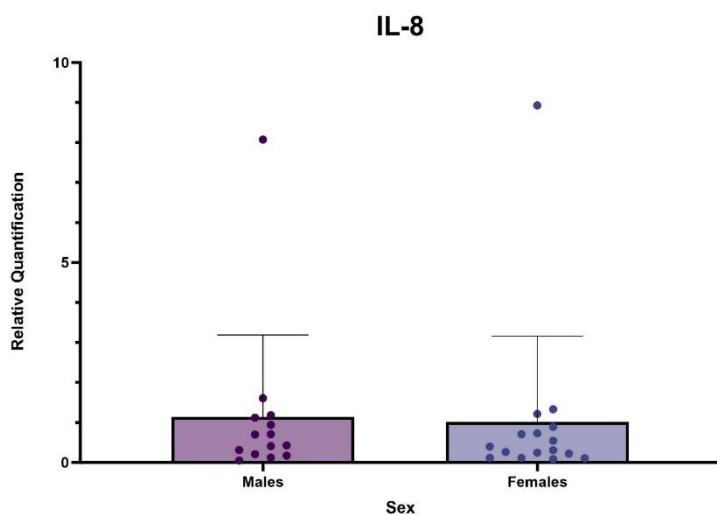


Figure 25: IL-8 relative expression across ages. Data includes both males and females. Each dot corresponds to a sample. N=8 (6, 14 and 20 weeks), N=6 (26 weeks); n=3.



The results revealed no significant impact of sex on the gene expression of IL-8 (figure 26).

Figure 26: IL-8 relative quantification of males compared to females. Each dot corresponds to a sample. N=14 (males) and N=16 (females); n=3.

Toll like receptor 2 (TLR2)

The expression of the pro-inflammatory TLR2 did not differ with age (figure 27). Expression is higher at 14 weeks compared to the other ages; however, significance is not reached due to the variability at this time point.

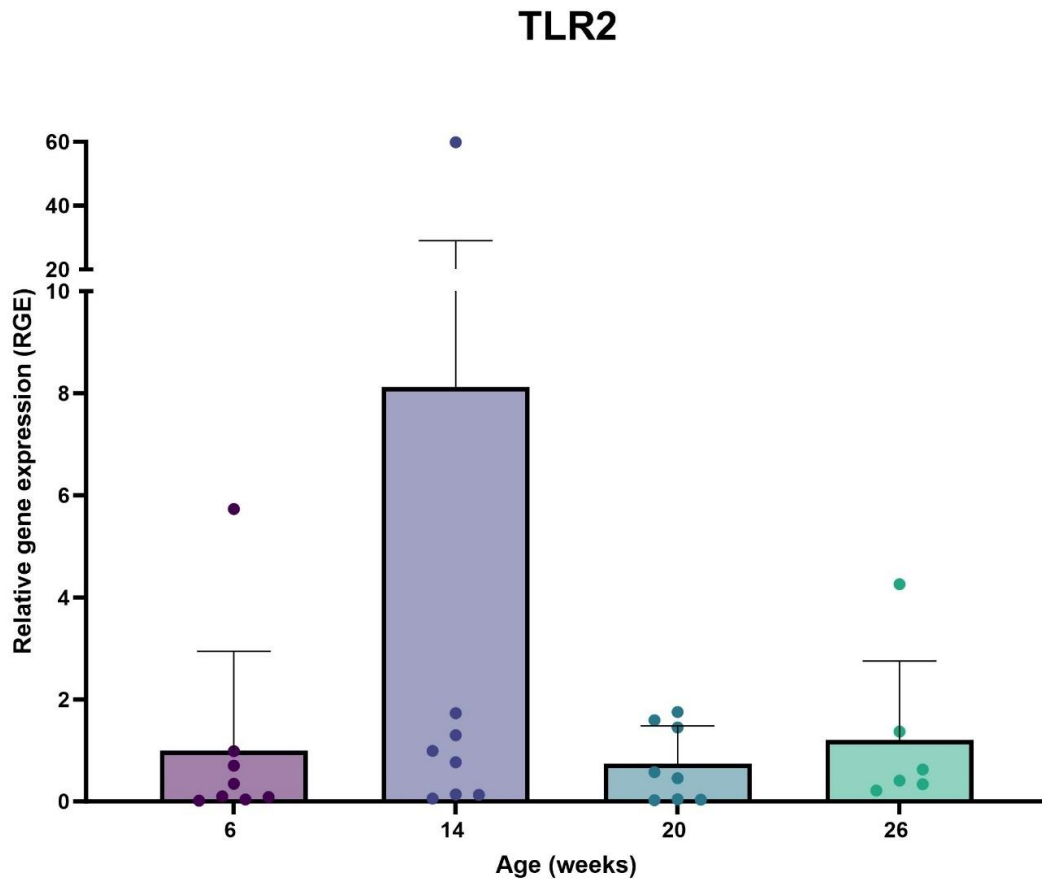
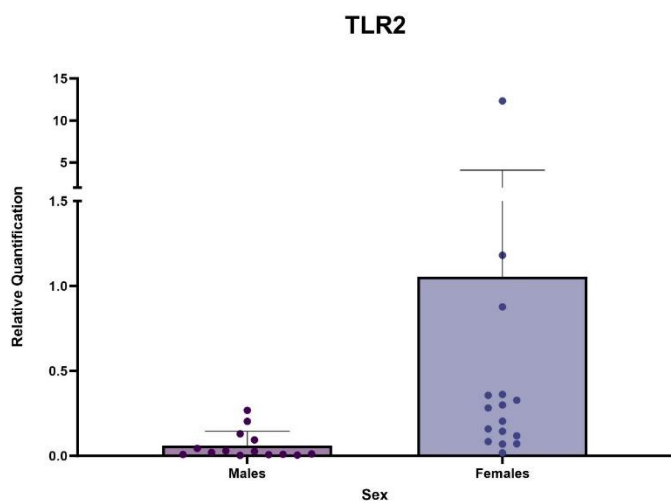


Figure 27: TLR2 relative expression across ages. Data includes both males and females. Each dot corresponds to a sample. N=8 (6, 14 and 20 weeks), N=6 (26 weeks); n=3.



The results revealed no significant impact of sex on the gene expression of TLR2 (figure 28).

Figure 28: TLR2 relative quantification of males compared to females. Each dot corresponds to a sample. N=14 (males) and N=16 (females); n=3.

2 Discussion

We hypothesized that with age, inflammation response would be dysregulated, leading to inflammaging within the liver of *Nothobranchius furzeri*. This dysregulation would induce a variation in the gene expression of cytokines, receptors and related molecules involved in the inflammatory process. We expected an increase in gene expression of the pro-inflammatory cytokines and receptors measured (IL-8, TLR2) and a decrease in gene expression of the anti-inflammatory cytokines and regulating molecules measured (IL-10; APOA-1).

IL-10 is considered one of the main anti-inflammatory cytokines; it is produced by almost all immune cells and inhibits many pro-inflammatory cytokines. Therefore, we expected its expression to decrease with aging. In contrast, we measured a significant increase from 6 weeks of age but plateauing between 14, 20 and 26 weeks of age. A high expression of IL-10 at an advanced age could indicate an increased requirement of IL-10 to combat a pro-inflammatory state. To confirm this, next steps could be to measure fibrosis, which is the end result of inflammation (39), through histology using Sirius red/fast green staining.

Previous studies reported that the expression of IL-10 with advanced age decreased in the retina and optic tectum of *N. furzeri* (figure 29) (21).

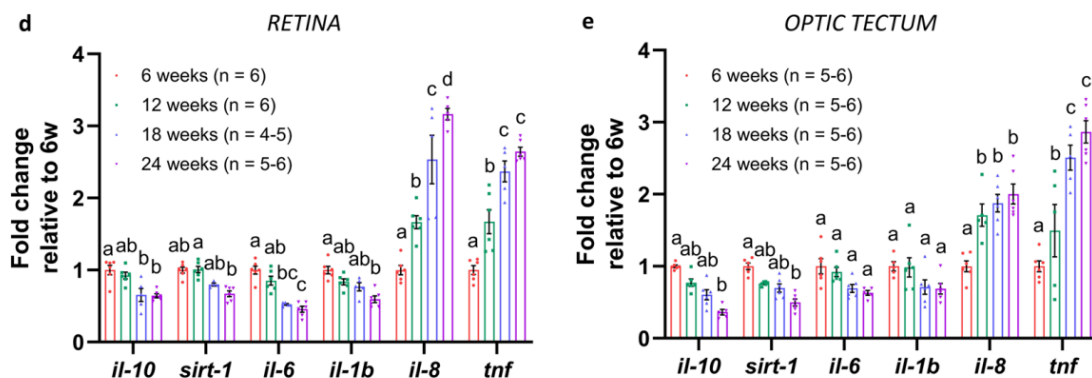


Figure 29: Gene expression of inflammatory markers in retinal and optic tectum of *N. furzeri* (21).

APOA-1 is an immune regulator that has anti-inflammatory properties as well as being involved in cholesterol homeostasis. TLR2 is a receptor within the PRRs family, involved in the detection of PAMPs and DAMPs, and is therefore essential for the start of the inflammation response. The stable expression across ages in the liver for both APOA-1 and TLR2 could signify that they are needed as importantly in young and advanced age individuals for their respective roles. To verify whether this is the case, current studies done by another student working in the lab are consisting of LPS injection within *N. furzeri* to determine if a strong immune stressor induce a different response between young and old fish. Also, next steps can include measuring the circulating levels of these cytokines using an ELISA. However, due to the size of the fish and difference in sequence specificity, ELISAs may not be a feasible option at this time.

IL-8 is a chemokine that is secreted by macrophages to recruit neutrophils, necessary for the inflammation response. Previously, the expression of IL-8 was reported to increase

with age in the central nervous system (figure 29) (21). In contrast, we did not detect any differences in the gene expression of IL-8 across the aging timepoints in the liver.

This study appears to be one of the first measuring gene expression of markers of inflammation within the liver of *N. furzeri*. Out of the four genes analyzed, only IL-10 expression was detected to be impacted by aging. These results differ from the *Vanhunsel et al.* (21), as they showed a decreased expression of IL-10 and an increased expression of IL-8 with aging within retina and optic tectum. The difference between these published data and those in the current experiments could be due to the tissues sampled. The retina and optic tectum are components of the central nervous system, whereas we sampled from liver tissues. The ages of the fish are also slightly different, but this is not expected to have caused such differences in the pattern of gene expression across the ages. In addition, differences may arise due to primers used. Although, the primer list was published in *Vanhunsel et al.* (21), we found that they did not meet our criteria for optimal primers. Therefore, we redesigned certain primers within the primer pairs, for example the forward primer for IL-10 and the reverse primer for IL-8 reverse (table 3). Nevertheless, some of our results agreed with those obtained by a previous master student work done within brain tissues of *N. furzeri* (40). Indeed, the results revealed no significant impact of age on gene expression of TLR2 and APOA-1 in brain in the same manner as we observed in liver. However, statistical analysis revealed an impact of sex on gene expression of APOA-1, in opposition with what we obtained. It is important to note that a lot of variability also appeared in the results of this study (40).

Regardless of the gene, we measured large variations within each age group, particularly at 14 weeks of age which was primarily due to one individual with elevated gene expression levels. Although an error may have been introduced during the sample manipulation, this was not detected at the level of mRNA. Furthermore, to explore if this variation impacted the results, statistical analysis was performed with and without some of these data points, yet the overall results did not change, and the differences remained insignificant.

It is important to note that the 30 samples used for these experiments are individually unique, therefore we are interpreting the impact of age and sex on different individuals. It would be interesting to follow the impact at multiple timepoints of the same individuals. This could be done by using another tissue, such as the tail fins, in which the tissue regenerates even in fish at older ages (41).

Through this master's thesis we measured gene expression across aging to better understand the impact of age on markers of inflammation. However, it is important to note that gene expression may not accurately reflect protein activity within a tissue as the correlation between mRNA and protein level is notoriously low in mammalian cells (42) (43). We found very few information on the same correlation within teleost. Despite this, mRNA expression is still commonly used in biological research.

Evaluating protein levels can be done with an ELISA test if sample size permits and specific antibodies are readily available. Unfortunately, the limited size of blood samples collectable from *N. furzeri* raises a challenge. Additionally, the antibodies used within an ELISA test may lack the required specificity to detect *N. furzeri* proteins.

Conclusion and perspectives

The results obtained through this master's thesis revealed an increase in gene expression of IL-10 with advanced age in the liver of *Nothobranchius furzeri*. This contrasts with our hypothesis as we expected a decrease in gene expression of anti-inflammatory markers, such as IL-10, across aging. In addition, analysis of gene expression of APOA-1, TLR2 and IL-8 showed no significant impact of age or sex. Differences may come from the high variability between samples of the 14-20 and 26 weeks of age. Moreover, the 26 weeks group is only composed of 6 samples compared to eight samples in the other groups, meaning its impact is less powerful when we analyze our graphs. A higher and equal sample pool for each group would always be more interesting.

This current study appears to be the first to measure gene expression of cytokines in the liver of *N. furzeri*. Therefore we compared our results with a similar experimental design observed in *Vanhunsel* article where they measured gene expression of cytokines in the central nervous system of *N. furzeri* (21). Our results contrast with theirs as they confirmed the up-regulation of pro-inflammatory cytokine IL-8 and down-regulation of anti-inflammatory cytokine IL-10 with aging. Differences could appear because we used a different organ model. Primers used were also different and designed differently to match our ideal primer parameters. Therefore, upcoming experiments could involve new primer design of the same genes tested in this thesis. In addition, some markers of inflammation have been selected, but many more markers exist, and their gene expression could be measured to validate or refute our hypothesis.

In the future, other techniques focusing on measuring protein levels or protein activity could be interesting. ELISA test is a well-known technique that could be used for that purpose, unfortunately limited size of blood samples in *N. furzeri* makes it currently unfeasible. A recent study in a preprint format used an alternative technique to measure inflammatory proteins in head kidney of *Nothobranchius furzeri* (44). Head kidney is a hematopoietic organ in *N. furzeri* and is like the adrenal gland in humans. The authors performed a Tandem Mass Tag Mass Spectrometry (TMT-MS) looking for systemic markers of immune aging and found that advanced age killifish (17 weeks) has a distinct proteome from the younger ones (7 weeks). Using Gene Ontology analysis, they were able to identify an enrichment in proteins involved in the inflammation process such as IL-1 β in aged individuals.

References:

1. Kanasi E, Ayilavarapu S, Jones J. The aging population: demographics and the biology of aging. *Periodontol 2000*. 2016;72(1):13-8.
2. Harman D. The aging process. *Proc Natl Acad Sci*. nov 1981;78(11):7124-8.
3. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The Hallmarks of Aging. *Cell*. 6 juin 2013;153(6):1194-217.
4. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. *Cell*. 19 janv 2023;186(2):243-78.
5. Rea IM, Gibson DS, McGilligan V, McNerlan SE, Alexander HD, Ross OA. Age and Age-Related Diseases: Role of Inflammation Triggers and Cytokines. *Front Immunol* [Internet]. 2018 [cité 15 avr 2023];9. Disponible sur: <https://www.frontiersin.org/articles/10.3389/fimmu.2018.00586>
6. Sameer AS, Nissar S. Toll-Like Receptors (TLRs): Structure, Functions, Signaling, and Role of Their Polymorphisms in Colorectal Cancer Susceptibility. *BioMed Res Int*. 12 sept 2021;2021:1157023.
7. Serhan CN, Chiang N, Dalli J, Levy BD. Lipid Mediators in the Resolution of Inflammation. *Cold Spring Harb Perspect Biol*. févr 2015;7(2):a016311.
8. Minciullo PL, Catalano A, Mandraffino G, Casciaro M, Crucitti A, Maltese G, et al. Inflammaging and Anti-Inflammaging: The Role of Cytokines in Extreme Longevity. *Arch Immunol Ther Exp (Warsz)*. 1 avr 2016;64(2):111-26.
9. Sethi G. TNF: A master switch for inflammation to cancer. *Front Biosci*. 2008;Volume(13):5094.
10. Zanni F. Marked increase with age of type 1 cytokines within memory and effector/cytotoxic CD8+ T cells in humans: a contribution to understand the relationship between inflammation and immunosenescence. *Exp Gerontol*. 1 sept 2003;38(9):981-7.
11. Tall AR, Yvan-Charvet L. Cholesterol, inflammation and innate immunity. *Nat Rev Immunol*. févr 2015;15(2):104-16.
12. Fearon WF, Fearon DT. Inflammation and Cardiovascular Disease. *Circulation*. 20 mai 2008;117(20):2577-9.
13. Wang WY, Tan MS, Yu JT, Tan L. Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Ann Transl Med*. juin 2015;3(10):136.
14. Shahzad A, Knapp M, Lang I, Köhler G. Interleukin 8 (IL-8) - a universal biomarker? *Int Arch Med*. 15 juin 2010;3(1):11.
15. Rose-John S. IL-6 Trans-Signaling via the Soluble IL-6 Receptor: Importance for the Pro-Inflammatory Activities of IL-6. *Int J Biol Sci*. 24 oct 2012;8(9):1237-47.

16. Li CX, Chen LL, Li XC, Ng KTP, Yang XX, Lo CM, et al. ApoA-1 accelerates regeneration of small-for-size fatty liver graft after transplantation. *Life Sci.* 15 déc 2018;215:128-35.
17. Wurfel MM, Kunitake ST, Lichenstein H, Kane JP, Wright SD. Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J Exp Med.* 1 sept 1994;180(3):1025-35.
18. Sziksz E, Pap D, Lippai R, Béres NJ, Fekete A, Szabó AJ, et al. Fibrosis Related Inflammatory Mediators: Role of the IL-10 Cytokine Family. *Mediators Inflamm.* 24 juin 2015;2015:e764641.
19. Kim DH, Jung IH, Kim DH, Park SW. Knockout of longevity gene Sirt1 in zebrafish leads to oxidative injury, chronic inflammation, and reduced life span. *PLOS ONE.* 6 août 2019;14(8):e0220581.
20. Hunt NJ, Kang SW (Sophie), Lockwood GP, Le Couteur DG, Cogger VC. Hallmarks of Aging in the Liver. *Comput Struct Biotechnol J.* 1 janv 2019;17:1151-61.
21. Vanhunsel S, Bergmans S, Beckers A, Etienne I, Van houcke J, Seuntjens E, et al. The killifish visual system as an in vivo model to study brain aging and rejuvenation. *Npj Aging Mech Dis.* 17 août 2021;7(1):1-17.
22. Schmauck-Medina T, Molière A, Lautrup S, Zhang J, Chlopicki S, Madsen HB, et al. New hallmarks of ageing: a 2022 Copenhagen ageing meeting summary. *Aging.* 31 août 2022;14(16):6829-39.
23. Vallabhapurapu S, Karin M. Regulation and Function of NF- κ B Transcription Factors in the Immune System. *Annu Rev Immunol.* 2009;27(1):693-733.
24. Koyama Y, Brenner DA. Liver inflammation and fibrosis. *J Clin Invest.* 3 janv 2017;127(1):55-64.
25. Morsiani C, Bacalini MG, Santoro A, Garagnani P, Collura S, D'Errico A, et al. The peculiar aging of human liver: A geroscience perspective within transplant context. *Ageing Res Rev.* 1 mai 2019;51:24-34.
26. Wilkins BJ, Pack M. Zebrafish Models of Human Liver Development and Disease. *Compr Physiol.* juill 2013;3(3):1213-30.
27. Poeschla M, Valenzano DR. The turquoise killifish: a genetically tractable model for the study of aging. Dickinson MH, Voshall LB, Dow JAT, éditeurs. *J Exp Biol.* 1 févr 2020;223(Suppl_1):jeb209296.
28. Kim Y, Nam HG, Valenzano DR. The short-lived African turquoise killifish: an emerging experimental model for ageing. *Dis Model Mech.* 1 févr 2016;9(2):115-29.
29. Hu CK, Brunet A. The African turquoise killifish: A research organism to study vertebrate aging and diapause. *Aging Cell.* 2018;17(3):e12757.

30. Valenzano DR, Terzibasi E, Cattaneo A, Domenici L, Cellerino A. Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish *Nothobranchius furzeri*. *Aging Cell*. 2006;5(3):275-8.
31. Genade T, Benedetti M, Terzibasi E, Roncaglia P, Valenzano DR, Cattaneo A, et al. Annual fishes of the genus *Nothobranchius* as a model system for aging research. *Aging Cell*. 2005;4(5):223-33.
32. Baumgart M, Di Cicco E, Rossi G, Cellerino A, Tozzini ET. Comparison of captive lifespan, age-associated liver neoplasias and age-dependent gene expression between two annual fish species: *Nothobranchius furzeri* and *Nothobranchius korthause*. *Biogerontology*. 1 févr 2015;16(1):63-9.
33. Barth E, Srivastava A, Stojiljkovic M, Frahm C, Axer H, Witte OW, et al. Conserved aging-related signatures of senescence and inflammation in different tissues and species. *Aging*. 12 oct 2019;11(19):8556-72.
34. Waits ER, Martinson J, Rinner B, Morris S, Proestou D, Champlin D, et al. Genetic Linkage Map and Comparative Genome Analysis for the Atlantic Killifish (*Fundulus heteroclitus*). *Open J Genet*. 4 mars 2016;6(1):28-38.
35. Vanhunsel S, Bergmans S, Beckers A, Etienne I, Van houcke J, Seuntjens E, et al. The killifish visual system as an in vivo model to study brain aging and rejuvenation. *Npj Aging Mech Dis*. 17 août 2021;7(1):1-17.
36. Ruijter JM, Barnewall RJ, Marsh IB, Szentirmay AN, Quinn JC, van Houdt R, et al. Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting. *Clin Chem*. 1 juin 2021;67(6):829-42.
37. Derveaux S, Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. *Methods*. avr 2010;50(4):227-30.
38. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 18 juin 2002;3(7):research0034.1.
39. Wynn T. Cellular and molecular mechanisms of fibrosis. *J Pathol*. janv 2008;214(2):199-210.
40. Van de Velde M. Brain inflammation linked to aging : using *Nothobranchius furzeri* as a model ? 2022.
41. Wendler S, Hartmann N, Hoppe B, Englert C. Age-dependent decline in fin regenerative capacity in the short-lived fish *Nothobranchius furzeri*. *Aging Cell*. 2015;14(5):857-66.
42. Silva GM, Vogel C. Quantifying gene expression: the importance of being subtle. *Mol Syst Biol*. oct 2016;12(10):885.

43. Koussounadis A, Langdon SP, Um IH, Harrison DJ, Smith VA. Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system. *Sci Rep.* 8 juin 2015;5(1):10775.
44. Morabito G, Dönertas HM, Sperti L, Seidel J, Poursadegh A, Poeschla M, et al. Spontaneous onset of cellular markers of inflammation and genome instability during aging in the immune niche of the naturally short-lived turquoise killifish (*Nothobranchius furzeri*) [Internet]. *bioRxiv*; 2023 [cité 10 janv 2024]. p. 2023.02.06.527346. Disponible sur: <https://www.biorxiv.org/content/10.1101/2023.02.06.527346v3>

Annex

Annex 1 : Samples information

Age	Sample	Sex	DOB	DOD	Size (cm)	Weight (g)
6 weeks	M6.1	M	01-12-20	14-01-21	2,9	0,41
	M6.2	M	01-12-20	14-01-21	3,2	0,48
	M6.3	M	01-12-20	14-01-21	3,3	0,54
	M6.4	M	01-12-20	14-01-21	3,1	0,45
	F6.1	F	01-12-20	14-01-21	2,8	0,24
	F6.2	F	01-12-20	14-01-21	2,4	0,15
	F6.3	F	01-12-20	14-01-21	2,8	0,3
	F6.4	F	01-12-20	14-01-21	2,5	0,2
14 weeks	M14.1	M	10-03-20	18-06-20	4,5	1,48
	M14.2	M	10-03-20	18-06-20	4,3	1,28
	M14.3	M	10-03-20	18-06-20	4,2	1,36
	M14.4	M	10-03-20	18-06-20	4,5	1,47
	F14.1	F	10-03-20	18-06-20	4	0,92
	F14.2	F	10-03-20	18-06-20	3,7	0,7
	F14.3	F	10-03-20	18-06-20	3,8	0,69
	F14.4	F	10-03-20	18-06-20	3,9	0,83
20 weeks	M20.1	M	10-03-20	28-07-20	4,2	1,26
	M20.2	M	10-03-20	28-07-20	4,6	1,61
	M20.3	M	10-03-20	28-07-20	4,6	1,97
	M20.4	M	10-03-20	28-07-20	4,5	1,48
	F20.1	F	10-03-20	28-07-20	3,7	0,67
	F20.2	F	10-03-20	28-07-20	4	1
	F20.3	F	10-03-20	28-07-20	4	1,28
	F20.4	F	10-03-20	28-07-20	4,1	1,24
26 weeks	M26.1	M	10-03-20	08-09-20	4,2	1,03
	M26.2	M	10-03-20	08-09-20	4,2	1,39
	F26.1	F	10-03-20	08-09-20	NA	NA
	F26.2	F	10-03-20	08-09-20	5	1,91
	F26.3	F	10-03-20	08-09-20	4	1,07
	F26.4	F	10-03-20	08-09-20	4	1,14

Annex 2: RNA concentrations for all samples

Age	Sample Name	Concentration (ng/ μ L)	Purity (A260/A280)	Purity (A230/A260)
6 weeks	M6.1	251.6	2.06	2.00
	M6.2	592.85	2.11	2.17
	M6.3	634.95	2.16	2.28
	M6.4	680	2.14	2.00
	F6.1	649.55	2.11	2.27
	F6.2	308.75	2.03	1.90
	F6.3	818.15	2.07	2.28
	F6.4	311.25	2.16	2.26
14 weeks	M14.1	1630.75	2.12	2.24
	M14.2	1729.2	2.12	2.31
	M14.3	962.5	2.01	2.28
	M14.4	890.4	2.06	2.28
	F14.1	416.8	2.05	2.19
	F14.2	1446.55	2.13	2.36
	F14.3	1219.7	2.11	2.25
	F14.4	1389.95	2.14	2.33
20 weeks	M20.1	1549.4	2.12	2.34
	M20.2	827	2.14	2.26
	M20.3	806	2.15	2.07
	M20.4	1803.45	2.14	2.31
	F20.1	1317.15	2.13	2.33
	F20.2	1444.45	2.13	2.33
	F20.3	1650.65	2.11	2.34
	F20.4	1343.15	2.12	2.38
26 weeks	M26.1	1103.75	2.13	2.36
	M26.2	1540.26	2.10	2.34
	F26.1	1934.4	2.11	2.33
	F26.2	1131.4	2.13	2.28
	F26.3	3409.65	2.11	2.35
	F26.4	2892.15	2.13	2.33

Annex 3: Example of 96-wells plate design

HPRT	1	2	3	4	5	6	7	8	9	10	11	12
A	M6.1	M6.1	M6.1	M6.2	M6.2	M6.2	M6.3	M6.3	M6.3	M6.4	M6.4	M6.4
B	F6.1	F6.1	F6.1	F6.2	F6.2	F6.2	F6.3	F6.3	F6.3	F6.4	F6.4	F6.4
C	M14.1	M14.1	M14.1	M14.2	M14.2	M14.2	M14.3	M14.3	M14.3	M14.4	M14.4	M14.4
D	F14.1	F14.1	F14.1	F14.2	F14.2	F14.2	F14.3	F14.3	F14.3	F14.4	F14.4	F14.4
E	M20.1	M20.1	M20.1	M20.2	M20.2	M20.2	M20.3	M20.3	M20.3	M20.4	M20.4	M20.4
F	F20.1	F20.1	F20.1	F20.2	F20.2	F20.2	F20.3	F20.3	F20.3	F20.4	F20.4	F20.4
G	F26.1	F26.1	F26.1	F26.2	F26.2	F26.2	F26.3	F26.3	F26.3	F26.4	F26.4	F26.4
H	M26.1	M26.1	M26.1	M26.2	M26.2	M26.2		No RT 14	6	14	20	26

Annex 4: Summary of best parameters for primers optimization and their efficiency

Genes	Primer ratio F/R	RNA quantity (ng)	Dilution serie	Efficiency (%)
Genes of interest				
TNF- α	NO	NO	NO	NO
IL-6	NO	NO	NO	NO
IL-10	500/500	1000	2x	101.85
IL-1 β	NO	NO	NO	NO
TLR2	250/500	1000	2x	88.12
APOA-1	500/500	1000	2x	99.85
IL-8	500/500	1000	2x	103.84
SIRT-1	NA	NA	NA	NA
Reference genes				
ACTB	500/500	1000	10x	92.29
HPRT	250/500	1000	10x	82.66
TBP	500/500	1000	5x	122.26
GAPDH	500/500	1000	10x	98.67
EF1 α	500/500	1000	10x	97.91
INSR	500/500	1000	10x	102.57

NO stands for non-optimized: multiple parameters were tested without success; NA stands for non-attributed: optimization process was not done

The effect of aging on liver inflammation: *Nothobranchius furzeri* as a promising model

Aging is defined as the natural and progressive accumulation of physiological changes over time, which lead to diseases and inevitably to death. With current improvements in the health care system, the average human lifespan has greatly increased. However, advanced age is correlated with the onset of several diseases such as metabolic syndrome, cancers, cardiovascular diseases, and neurodegenerative diseases.

Aging has been defined with twelve hallmarks, one recently added hallmark is chronic inflammation. Although inflammation is a natural defense mechanism, if it is not resolved it can lead to chronic inflammation that can negatively impact immune cells. A new term has emerged to describe this chronic age dependent inflammation: inflammaging, which has been defined as a dysregulation of the inflammation response and is characterized by an increased production of pro-inflammatory cytokines accompanied with a decreased production of anti-inflammatory cytokines. For example, key pro-inflammatory cytokines involved in inflammation include TNF- α , IL-8, IL-1 β , IL-6 and TLR2, whereas key anti-inflammatory cytokines include IL-10, APOA-1 and SIRT-1.

Nothobranchius furzeri are a relatively new animal model to examine the underlying molecular and cellular mechanisms of aging. Results have begun to emerge that markers of inflammaging have been detected in the optic system of *N. furzeri*. We wish to further explore whether the expression of the above cytokines is impacted by advanced age in other tissues of *N. furzeri*. Previously, we found little evidence that age altered the expression of these genes in whole brain tissue homogenates. The aim of this present thesis is to address whether the same is true or not for liver tissue, another tissue that is susceptible to age-induced inflammation.

Real time-qPCR experiments were conducted on eight genes associated with inflammation (IL-10, TLR2, APOA-1, IL-8, TNF- α , IL1- β , SIRT-1 and IL-6) across four ages (6, 14, 20 and 26 weeks). Out of the eight genes, we were able to measure gene expression across aging for four of them: two anti-inflammatory molecules IL-10 and APOA-1, as well as two pro-inflammatory molecules TLR2 and IL-8.

The expression of IL-10 was significantly up-regulated at 14, 20 and 26 weeks of age compared to 6 weeks. In contrast, we did not detect any differences in gene expression of IL-8, TLR2 and APOA-1 across the four ages. Gene expression was also not impacted by biological sex.