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Department of Veterinary Sciences
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Evaluation of the activity of the immune system and age-related tissue markers in Turquoise killifish (*Nothobranchius furzeri*, Jubb 1971) and their role in cell ageing

Candidate
Dr. Emiliano Di Cicco

Tutor
Prof. Giacomo Rossi

SUMMARY

ABSTRACT

Currently the Turquoise Killifish is considered the best animal model suitable for aging research. This annual fish, from south east Africa, shows an exceptionally adaptive behaviour to dry periods: indeed, due to this extreme environmental characteristics, the life cycle of *Nothobranchius furzeri* is very fast, with an average lifespan of just about 8-9 weeks, making this species (more similar to highly developed vertebrates than nematodes or fruit flies) highly practical for aging studies.
The present study has evaluated the activity of the immune system as well as the expression of AGE-RAGE system, cell-damage related proteins (Bcl2, p53), mitosis activity marker (PCNA), and pro-apoptosis activity by T.U.N.E.L. method on the liver of four lifespan-specific strains of Turquoise Killifish (Nothobranchius furzeri, Jubb 1971), correlating the results with aging processes and tumor incidence. Some groups underwent caloric restriction in order to module their expected lifespan.
The results demonstrated an increase of age-related lesions along with the age in all the strains tested, due to a decrease of cellular-turn-over. This aspect was also influenced by the strain of the fish: longest lifespan strains showed later the similar lesions than short lifespan strains. Moreover caloric restriction groups showed lower incidence and severity of hepatic degeneration than control groups. Furthermore, there was a linear correspondence between the age of the model and its expected lifespan with the incidence and severity of neoplasm. The same relationship could be found in the expression of cell-damage related proteins (p53, Bcl2), age-related markers (AGE-RAGE system) and pro-apoptosis activity, as well as in the development of neoplasms. These results demonstrated the high feasibility of this fish as an excellent model to study the effects of aging processes and cancer genesis.

Keywords: ageing, tumorigenesis, liver, Nothobranchius

1. INTRODUCTION

1.1 AGEING

In biology, AGEING stands for the sum of irreversible modifications occurring on the organisms along with the lifespan and eventually leading to the death.

To date, there are three biological theories concerning ageing processes:

1- genetic theory: ageing represents a pre-established or genetically-programmed phase of the life as other ones (for instance, puberty) and hence it is regulated by modifications affecting some genes.
2- Homeostasis theory: ageing is the result of the progressive lack on the efficiency of neuro-endocrine and immune systems.
3- cellular damage theory: the environment greatly influences the organisms.

1.1.1 Genetic theory of Ageing

Different researches carried out on yeast (*Saccharomyces spp.*), nematodes (*Caenorhabditis elegans*) and fruit fly (*Drosophila melanogaster*) demonstrated several genes are involved on ageing and longevity mechanisms (Benguria et al., 1999; Wood at al., 2004; Kang et al., 2002). These genes often interact each others following a complicated network of connections. For instance, p66 gene is associated with an increased resistance to oxidative stress and codify an adaptive protein involved on the process of cell signalling. When experimentally deleted on rodent models, p66 results directly correlated with a reduction of cellular apoptosis, increasing the lifespan (Migliaccio et al., 1999).

1.1.2 Homeostasis theory of Ageing

The most important ageing indicator considered by this theory is the status of neuro-endocrine and immune systems.
Rudman et al. (1990) proposes ageing as the expression of a progressive neuro-endocrine dysfunction. The delicate biological mechanism affecting hormonal regulations may loose efficacy along the time, heading to alterations on organs and apparatus.
Immunological theory considers the reduction on the efficiency of the immune system as the main reason for ageing processes. This failure may lead to an increased predisposition to infectious diseases. In effect, the progressive decay of the immune response occurring along the lifespan is well known (Franceschi et al., 2006): T lymphocytes tent to lessen their activity, while several immune organs (such as bone marrow, spleen and lymph nodes) get strongly reduced or even disappear.

1.1.3 Cellular damage theory of Ageing

Ageing may be caused by the accumulation of different agents (such as free radicals) which gradually damage the cells inducing their degeneration (Colomba et al., 2005).

1.1.4 Basic concepts and mechanisms over cellular damage

Although potentially lethal to the cell, cellular damage may have different consequences as:

- temporary failure of a cellular function
- structural lesion causing permanent functional failure
- DNA lesion heading to hereditary alterations of cellular phenotype (mutation)

Plasmatic membrane, nucleus, mitochondria, endoplasmatic reticulus and lysosomes are feasible targets and primary sites of cellular damage. It may be induced by several substances or even their chemically active metabolites: such substances can be reduced by some reducing cellular agents, according to the oxidant potential of the substance. Moreover, other cellular substances such as

reduced glutatione (GSH) may act both as nucleophilic and reducing agent. Nucleophilic agents link covalently nucleophilic cellular substances, inducing arilation or alchilation of the proteins. Cellular reduttasi enzymatically reduce some substances by adding one or two electrons. When featured with redox activity, they may act as electron's donor, inducing the reduction of molecular oxygen and other critical substances inside the cell. Redox cycle of substances like menadione induces oxidative stress to the cell by producing a large amount of oxygen reactive products (primarily, the superoxide anion O_2). Such a process is included among those cellular damage mechanisms induced along with pathological situations associated to an increased production of oxygen reactive intermediates. These intermediates are called ROS (Reactive Oxygen Species) and originate by intracellular redox reactions involving molecular oxygen as final electron acceptor. Other than the superoxide anion (O_2), the most important ROS are the hydroxilic radicals and the hydrogen peroxide, all recognized as the principal mediators of cellular damage. The largest amount of intracellular ROS production occurs inside the mitochondria and is strictly associated to the metabolic status and the oxygen consumption. Moreover these intermediates react each others and with other molecules available inside the cell generating new molecules which may cause further cellular damages.

Oxidative stress is a condition of cellular damage characterized by an imbalance among ROS production and cellular antioxidant systems as:

- antioxidant substances as α-tocopherol, carotenoids and glutatione: small food-derived molecules acting not-enzymatically by linking the ROS
- enzymatic systems as superoxide-dismutase (SOD, which speeds up the conversion of superoxide to hydrogen peroxide), glutatione-peroxidasi (which converts the hydrogen peroxide to water)

Therefore, the imbalance between ROS production and elimination influences the degree of intracellular oxidative stress. In fact, several studies carried out in different species demonstrated the treatment with resveratrol (a polyphenol found in red wine) as well as the over-expression of antioxidant genes induce a longer lifespan (Barger et al., 2008; Bass et al., 2007), supporting the idea that oxidative damages induced by free radicals (ROS) tent to accumulate along with the lifespan and then represent the main responsibles for cellular ageing.

Both invertebrates and mammals show a longer lifespan strictly related to a higher resistance to oxidative stress (Finkel et al., 2002). A direct comparison on the (relative) lifespan among different species also demonstrated a shorter lifespan correlates with a higher metabolic rate, while inversely species showing longer lifespan have lower metabolic activity. These data may head to the hypothesis that the metabolic rate influence the lifespan as well. In effect the metabolic activity and the ROS production by mitochondria are strictly related and overall these two theories are often associated each other. Nevertheless a comparison on ROS production by mitochondria isolated from two taxonomically related rodents, the white-footed mouse (*Peromyscus leucopus*) and the house mouse (*Mus musculus*), which show a more than twofold difference in maximum lifespan potential (8.2 and 3.5 yr, respectively) presented contrasting results: despite *P.leucopus* has a higher metabolic rate, it also shows a longer lifespan, while the shorter-lived *M.musculus* produces more free radicals(Labinskyy et al., 2009), demonstrating the amount of ROS produced represent the main longevity indicator.

A prolonged exposure to free radicals may induce alteration to DNA, lipids and proteins. The cellular damage induced by the ROS consists in an increased permeability and deformity on the membranes (affecting particularly the mitochondria), inhibition of cationic pump (leading to modification of intracellular ionic homeostasis), ATP exhaustion and increase of free cytosolic Calcium (from mitochondria to cytoplasm). Mitochondrial DNA is more sensitive to free radical accumulation than nuclear DNA, and hence the ROS-induced damages are much more difficult to repair.

Along with the ageing process, there is an increased amount of oxidative damages occurring to the protein and above all to the lipids, leading to alteration of integrity and functionality of the cell

membrane. In fact free radicals induce a chain of reactions called lipoperoxidation, originating from membrane lipids and conducting to the production of lipid radicals, which get converted to peroxide lipid radicals in presence of oxygen. These molecules may attach adjacent fatty acids by removing them a hydrogen atom and generating lipid hydroxides, and contemporary producing new radicals which can propagate the process. Therefore this reaction among peroxide radicals with other lipids may start up an auto-catalytic process characterized by a sequence of peroxidation leading to the consumption of membrane lipids. Lipid peroxidation is particularly harmful due to two reasons:

1- membranes undergo a molecular damage inducing the inactivation of enzymes, biochemical defects and structural alteration leading the cell to the death
2- lipoperoxidation induces the production of water-soluble substances as aldeids, very aggressive towards several cell molecules and structures, which easily spread everywhere inside the cell, diffusing the cell damage far away the primitive site of oxidation. (Marcato P.S., 2000)

1.1.5 Lipofuscins (LF)

Lipofuscins are one of the several substances produced by lipid peroxidation, coming out from inside the secondary lysosomes due to the activity of ROS. Together with the lipochromes, they represent lipid-pigments, despite the latter are exogenous and plant-derived. LF are the result of a gradual oxidation and following polymerization of unsaturated lipids, due to failure of intracellular metabolism (peroxidation damage) of free fatty acids and glycerol-linked fatty acids.
Microscopically, LF are yellow-brown granules, often jaline. They are composed by a conglomerate of lipids, metals, organic molecules and biomolecules commonly showing auto-fluorescence at 360-470nm under UV light. The auto-fluorescence seem due to the aldeid polymerization produced by progressive lipid oxidation. LF granules are relatively insoluble in common lipid solvents and are stained by Black Sudan, Sudan III or Osmic acid. This feature allow them to get distinguished by other pigments as melanin or hemosiderin. LF are commonly known as “usury pigments” due to their accumulation easily detectable on atrophic cells and organs, in particular during senility or weakening diseases: they usually accumulate on myocardic fibers, epatocytes, renal epitheliums, nervous cells. Atrophic organs, especially heart and liver, show a brownish colouring (called “brown atrophy”) when undergo a strong accumulation. LF granules have been found on all the Eukaryotes, and due to the fact they tent to accumulate during the ageing process, they are also called “ageing pigments”. This phenomenon seems based on protract lack of antioxidants leading to an increase of catabolic reactions of intracellular lipids. Despite of that, lipofuscins are not considered harmful for the cell. (Marcato P.S., 2000)

1.1.6 Advanced Glycation End products (AGEs) and their receptors (RAGE)

Advanced Glycation End products are a heterogeneous group of glycosylated proteins which accumulate during aging processes and play an important role in the pathogenesis of a variety of chronic diseases as well as in cancerogenesis. In effect they are the result of a chain of chemical reactions after an initial glycation reaction. The intermediate products are known, variously, as Amadori, Schiff base and Maillard products, named after the researchers who first described them. Side products generated in intermediate steps may be oxidizing agents (such as hydrogen peroxide), or not (such as beta amyloid proteins) (Miyata et al., 1993).
AGEs may be formed external to the body (exogenously) by heating (e.g., cooking) sugars with fats or proteins (Koschinsky et al., 1997), or inside the body (endogenously) through normal metabolism and aging. Under certain pathologic conditions (e.g., oxidative stress due to hyperglycemia in patients with diabetes), AGEs formation can be increased beyond normal levels. The formation and accumulation of AGEs has been implicated in the progression of age-related diseases (Tan et al., 2006). They are recognized as photosensitizers in the crystalline lens through crosslinking (Fuentealba et al., 2009) which has implications for cataract development (Gul et al.,

2009). AGEs have been implicated in Alzheimer's Disease (Srikanth et al, 2009) cardiovascular disease (Simm et al., 2007), and stroke (Zimmerman et al., 1995). The mechanism by which AGEs induce damage is through a process called cross-linking that causes intracellular damage. AGEs may be less, or more, reactive than the initial sugars they were formed from. They are absorbed by the body during digestion with about 30% efficiency. Many cells in the body (for example, endothelial cells, smooth muscle or cells of the immune system) from tissue such as lung, liver, kidney or peripheral blood bear the Receptor for Advanced Glycation End products (RAGE) that, when binding AGEs, contributes to age- and diabetes-related chronic inflammatory diseases such as atherosclerosis, asthma, arthritis, myocardial infarction, nephropathy, retinopathy, or neuropathy. There may be some chemicals, such as aminoguanidine, that limit the formation of AGEs (Wells-Knecht et al., 1995).

AGEs affect nearly every type of cell and molecule in the body, and are thought to be one factor in aging and some age-related chronic diseases (Glenn et al., 2009; Semba et al., 2009a; Semba et al., 2009b). They are also believed to play a causative role in the vascular complications of diabetes mellitus (Yan et al., 2007a).

They have a range of pathological effects, including increasing vascular permeability, inhibition of vascular dilation by interfering with nitric oxide, oxdising LDL (Gugliucci & Bendayan, 1996), binding cells including macrophage, endothelial and mesangial cells to induce the secretion of a variety of cytokines and enhancing oxidative stress (Gugliucci & Bendayan, 1996; Yan et al., 2007b) and apoptosis (Shaikh and Nicholson, 2008). Reduced muscle function is also associated with AGEs (Haus et al., 2007).

RAGE are multiligand receptors of the immunoglobulin superfamily of cell surface molecules that binds molecules that have been irreversibly modified by non-enzymatic glycation and oxidation as AGEs but also act as counter-receptors for HMGB1, S100/calgranuline and β-amiloid peptides. Interactions with these ligands activate important cell signalling pathways leading to the production of pro-inflammatory cytokines.

1.2 ANIMAL MODELS TO STUDY AGEING PROCESSES

Several species have been used to study ageing and age-related diseases so far, depending not only on scientific issues, but also on practical and economic considerations.

Invertebrates have been widely utilized, due to the short lifespan and easy maintenance in captivity. The fruit fly (*Drosophila melanogaster*) easily satisfy these requirements: it is small in size, easy to keep and prolific, and show a short lifespan. These features allow to obtain a large number of specimens to carry out several studies. Moreover, this model can be transgenically modified, allowing studies on the up- or down-regulation of several genes involved on ageing processes as well as the comprehension of the mechanisms underlying the acetilation of the histons and the regulation of the genes expression (Kang et al., 2002). However, the ongoing selection among the different *Drosophila* strains may induce heterosis on the lifespan: the offspring tent to live longer, making more difficult the evaluations of the results.

Another model, the self-fecundating hermaphrodite nematode *Caenorhabditis elegans*, showed no heterosis effect on selective strain crossing, in particular between Bergerac and Bristol strains. In fact this model have been used to evaluate how much the genetic heredity may affect the lifespan rather than the environment (Wood at al., 1982). *C. elegans* have also been widely used in gerontology, despite of the lack of cellular divisions on adult somatic cells (post-mitotic cells), to identify some new drug affecting the lifespan: it is well known the degenerative processes related with ageing go along with the same pathways followed by the Vertebrates. Ethosuccimide, trimethadione and 3,3-dietil-2-pyrrolidone are only a few of these drugs. Low keeping temperature also affects the lifespan in *C. elegans* as well as in *Drosophila*, making it longer (Evason et al., 2005).

Despite of these advantages, invertebrate models show several morphological, histological and physiological differences to the Vertebrates, making difficult the applications of these observations to the Humans. Moreover, having only post-mitotic cells, they can not be utilized to carry out studies on cell replication along with ageing. For these reasons, a mammal model has been demanded, and to date the murine model is the most employed.

In Mammals, a longer lifespan has been correlated with a higher resistance to oxidative stress (Barger et al., 2008). First demonstration was the study carried out on mice with a mutation on the protein p66shc (Migliaccio et al., 1999) which showed longer lifespan. Embryonic mouse fibroblasts (knock-out strain) with a deletion of protein p66shc resulted more resistant to apoptosis induced by oxidative stress than wild-type cells (Migliaccio et al., 1999). Moreover this protein seems to regulate the ROS production, correlating this aspect with the lifespan in mammals (Finkel et al., 2000).

Nevertheless the murine model shows some disadvantages as well: their lifespan is too long (about 2-3 years) to be suitable for ageing researches. Moreover it requires an accurate and continuous monitoring of the environmental conditions in order to avoid the transmission of infectious diseases. Despite of that, a large number of specimens dies along with the studies before reaching the desired age. To obtain a 24 to 36 months old mouse it is required to keep at least 3 specimens. In 1970 a new strain (SAM – Senescence Accelerated Mice) characterized by precocious ageing (6 months) was selected, then a large number of strains were created. All these derived strains are now characterized by strict uniformity (essentially homozygote) due to accurate breeding programs, in order to eliminate genetic variability. However, this is an unnatural condition: several harmful genes are recessives and hence can act only in homozygosis. Along with the inbreeding process, several genes are lost for natural selection, while others get fixed in less resistant animals. The latter specimens don't represent the wild-type because they miss a part of their genetic pool. Even new wild specimens will be less representing along with the time because they will get adapted to the lab environment (McClearn, 1997). Considering overall ageing studies are carried out by comparing the results between lab strains and wild-type, this situation is not desirable.

1.2.1 Fish models for ageing studies

Fish may represent a fitting compromise between invertebrates and mammals. There are several species suitable for ageing studies. In general they are small in size, easy to keep and very prolific, allowing to maintain a large number of specimens in small rooms with less time and money waste. Ageing pathways occurring in fish are comparable to other vertebrates: oxidative stress acts as ageing source, antioxidant enzymes carry out a protective rule, the ability to repair DNA damages decreases along with the age and finally caloric restriction delays ageing (Kishi et al., 2003; Malek et al., 2004).

The first fish utilized for ageing studies have been the zebrafish (*Danio rerio*): living as much as the mice, and having all the above mentioned features, zebrafish is an appreciated animal model (Liu et al., 1975; Yen at al, 2004). Moreover, the lifespan of this fish can be prolonged acting on several different parameters:

- there are selected long-lived inbreed strains
- the specimens may undergo caloric restriction
- a recycling water filtration system may affect the lifespan (due to a "standardized" environment)
- low keeping temperature delays the comparison of age-related pathologies (Valenzano et al., 2006d)

Despite of all these advantages, only 50-60% of zebrafish embryos reaches the adultness (Herrera et al., 2004). Moreover, there are no advantage respect the murine model regarding the lifespan.

To resolve this problem, the use of killifish (*Nothobranchius sp.* in particular) has been proposed: this group of fish may finally meet all the requirements for ageing studies.

Nothobranchius species are popular aquarium fish from East Africa, small in size (about 4-5 cm long), relatively easy to keep and maintain in captivity due to a rapid growth and good prolificacy. Moreover, the development of the eggs can be modulated by temperature and the embryos can be economically stocked for a fair amount of time. Nevertheless, research facilities must be regularly monitored to avoid the spreading of infectious diseases (Genade at al., 2005).

The lifespan of Nothobranchius species is relatively short, depending on the different permanence and duration of the rainy season in each habitat. The lifespan may vary significantly even among different strain of the same species (e.g. *N. furzeri*), due to the particular nature of the ponds and the on-site rainy precipitation. As example, *Nothobranchius rachovii*, originating from Beira Mozambique), inhabits an area characterized by a sharp-cut distinction between dry and rainy season (annual rain precipitation: about 1600mm), and shows a lifespan of just 5-6 months, up to 9. Differently *Nothobranchius guentheri* originates from Zanzibar area (Tanzania), a wetter location placed within the tropical rainforest area which often doesn't get completely dry during the dry season: occasionally some pools may even connect with adjacent permanent rivers. Due to such a condition *N. guentheri* shows a longer lifespan, up to 16 months. On the opposite, Gona-Re-Zhou (GRZ) strain of *N. furzeri*, living in temporary ponds in South East Zimbabwe, the aridest area of the African subcontinent, shows a lifespan of just 8-11.5weeks. Such a variability is hence due to an adaptive behaviour to all these extreme environmental conditions.

Although the expected lifespan may vary greatly, the genetic differences occurring among all the Nothobranchius species are quite small. The presence of such a large amount of species (43, but more have not been classified or even discovered yet) and strains genetically correlated each others offers a rich material for comparative studies (Gerhard et al., 2004). Interestingly, Nothobranchius species show a accelerated growth associated with a premature expression of ageing biomarkers, making these models a delightful tool for ageing researches. As example, some genes regulating the progression of ageing processes are expressed in any terrestrial species, others only in mammals. However both p66shc protein and MTP (Microsomal Triglyceride Transfer Protein) amino acid sequences in Nothobranchius species are very similar to Humans, and the corresponding genes can be easily isolated. Therefore these fish might be used to test the effects of pharmaceutical compounds targeting specific genes of Vertebrates, taking advantage of the briefness of they

lifespan. Another marker for cellular senescence (SA-β-gal, Senescence associated β-galattosidase), already observed in zebrafish and Human, is over-expressed when *N. furzeri* is about 9 weeks old, demonstrating the rapid induction of cellular senescence is strictly correlated with their short lifespan (Genade at al., 2005). Finally, the decrease of locomotor activity along with the age is a marker of neuro-muscular ageing.

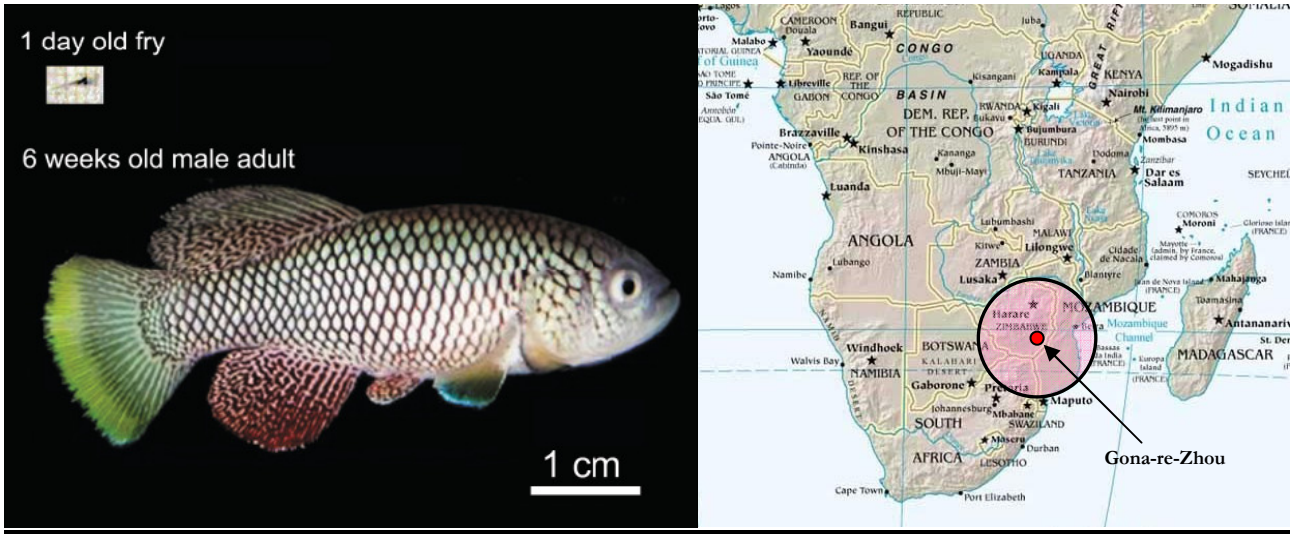
As above mentioned, Nothobranchius species are quite prolific: as example, *N. furzeri* reaches the sexual maturity when one month old, and then may lay 20-30eggs every day. Therefore it's easy to obtain a large amount of specimens in a short amount of time and, more importantly, it' s possible to carry out a complete and accurate demographic analysis of an experimental population in just few months.

As in zebrafish, there are different parameters which can be used to affect the lifespan of killifish:

- different species (and even different strains for the same species) show different lifespan
- caloric restriction prolongs the lifespan
- low keeping temperature prolongs the lifespan
- recycling water filtration decreases the lifespan (unlike zebrafish)

The latter parameter seems due to the attitude of zebrafish to inhabit rivers characterized by consistent flow, while Nothobranchius species usually reside in seasonal ponds with standing water.

1.3 NOTHOBRANCHIUS FURZERI – BIOLOGY



Nothobranchius furzeri (Jubb 1971) inhabits an area comprised among Zimbabwe, Mozambique and South Africa, which corresponds to the aridest region of the African subcontinent, with just 360mm of rain per year. The extremely short lifespan of this fish is due to the adaptation to this particular environment, characterized by a 10 months long dry season. For this reason the dry-resistant eggs require a dry period, in order to complete the embryonic development.

N. furzeri lives in small temporary ponds and pools created by the rain fallen during the short rainy season. The quality of the water is generally poor, and undergo significant temperature oscillations. In this environment the eggs laid on the bottom of the ponds during the previous rainy season hatch: the fries are about 6-7mm long and grow rapidly, feeding avidly on small invertebrates and crustacean at first, then on shrimps, worms and larvae of insects. The fish get sexually mature in few weeks (about 4 weeks) and reach the adultness when 6 weeks old. The sexual dimorphism is evident: the males are big and colourful, while the females are generally greyish, colourless and smaller in size. The males tent to be aggressive each other, and quite territorial. An adult female may lay 20-30 eggs per day (1mm in diameter), and several hundreds along with the lifespan, although old females are less prolific. The ponds get dry in few weeks: the adult fish die while the eggs, laid one-by-one on the muddy bottom, stay there waiting for the next rainy season or, in some

cases, even a few year. During this period, the eggs stay in a dormant phase called diapause, which blocks the development of the embryos. This is characterized by three stages:
- DI, with the completion of blastogenesis
- DII, with the completion of embryogenesis
- DIII, with the completion of the organogenesis

At the end of DIII, the fries are completely developed and wait for the right environmental conditions to hatch. At that moment, it's easy to note the eyes of the fry through the opaque shell of the eggs.

1.4 IMMUNE SYSTEM OF FISH

All fish lack bone marrow as well as lymph nodes. Lymphoid and myeloid tissue are more intermingled in fish, commonly associated with the kidney. Moreover, they possess a well-developed thymus wherein lymphocytes are the predominant population, although they are not organized into a distinct cortex and medulla. The spleen divided in red and white pulp as in mammals but the latter lacks germinal centres.

1.4.1 Lymphoid and myeloid tissue

A unique feature of piscine lymphomyeloid tissue is the presence of melanomacrophage centres in the liver, spleen and kidney. These aggregates of reticular cells, lymphocytes, macrophages and plasma cells may be considered the forerunners of the germinal centres present in the spleen and lymph nodes of higher vertebrates (Agius, 1985). The melanomacrophage centres are also repositories of pigments as lipofuscin, melanin and hemosiderin, which may occur contemporary in the same macrophage. Lipofuscins represent the result of the oxidation of unsaturated lipids. Melanin is commonly present at variable levels and seems to be capable of neutralizing free radicals and cation activity associated with oxidizing conditions. This would explain its close association with lipofuscin in macrophages and its increased accumulation infections and injuries. Hemosiderin, a by-product of haemoglobin degradation, is abundant in fish spleen under condition of starvation and disease (Agius, 1985). The role of melanomacrophage centres for modulating infections is speculative. The pigments may have a direct bactericidal effect (Edelstein, 1971) or may simply accumulate as a result of tissue damage at the site of infection, thereby enlarging the melanomacrophage centres. In fact, they develop focally in association with late stages of the chronic inflammatory response to severe tissue and in association with the cellular response to a variety of infections (Agius &Roberts, 2003). Moreover Press at al. (1996) demonstrated the retention of antigens and consequent activation of macrophages in the melanomacrophage centres is of vital importance to achieve immunological memory against bacterial antigens.

1.4.2 Phagocytosis

Phagocytosis is the most primordial defence mechanism: it involve recognition and attachment of a foreign particle, engulfment and digestion. Signals for recognition and attachment are largely unknown in fish, while kidney and spleen are the major sites of antigen localization by phagocytic cells. In fish there are two king of phagocytic cells: monocytes and macrophages, actively phagocytic mononuclear cells which opsonise or enhance the phagocytosis via antigen-antibody complexes by Fc receptors for binding antibody (Haynes at al., 1988), and granular leukocytes, including heterophils and eosinophils. Heterophils are the predominant granulocytes in fish blood but they are not actively phagocytic as mononuclear cells, but rather they kill extracellularly through the discharge of their hydrolytic and oxidizing enzymes. Eosinophils are also believed to be phagocytic cells (Mainwaring and Rowley, 1985).

1.4.3 Humoral immunity

Through the complex interaction of antigen-presenting cells and interleukins, B lymphocytes are stimulated to produce immunoglobulins. Interleukin 1 (IL-1), which is produced by macrophages after the exposure to antigen, activates helper T lymphocytes which in turn produce interleukin 2 (IL-2), supporting a proliferation of lymphoblastic cells., then stimulating B lymphocytes to produce immunoglobulins. Fish synthesize only one class of immunoglobulin equivalent to mammalian IgM. Serum IgM in teleosts is tetrameric, with epitopes on **μ** chains highly conserved and shared with other vertebrates. IgM is the major immunoglobulin isotype produced in a primary immune response and , despite IgG are absent in fish, protective humoral response occur. In fact IgM is more efficient in complement activation, opsonisation, neutralization of viruses and agglutination (Tizard, 1987). Secretory immunity, as opposed to systemic immunity, refers to a localized production of antibodies at mucosal surface and is associated with IgA isotype in

mammals, which primarly acts preventing the adherence of microorganisms. In teleosts, antigen-specific antibodies occur in mucus as IgM (St. Louis-Cormier et al., 1984; Lobb, 1987).

1.4.4 Cell-mediated immunity

In addiction to humoral immunity, T lymphocytes, originating from thymus, mediate cellular immunity. Different subsets regulate different functions. Helper T cells enhance, whereas suppressor T cells downregulate, the response of t and B lymphocytes. Cytotoxic T cells destroy foreign and abnormal cells. Another subset of T lymphocytes mediates delayed-type hypersensitivity reactions. Bony fishes have heterogenic T lymphocytes, although no functional markers distinguish the various subsets. Besides, bony fishes have acute allograft rejections in vivo and strong mixed lymphocytes reactions, demonstrating the existence of major histocompatibility complex (MHC), although both I MHC and II MHC have not been characterized yet.

1.4.5 Nonspecific immunity

Host defence mechanisms not involving specific recognition of antigen also occur in fish and are considered to be nonspecific, on the way that limit the spread or remove the cause of host-tissue damage (e.g. phagocytosis). The skin represents a physical barrier to invading microorganisms, while the skin mucus, acting as an additional external barrier, inhibits the colonization of microorganisms on the integument as well as on the gills and gastrointestinal mucosa: indeed mucus contains natural IgM, lysozyme and bacteriolysins (Diconza, 1970; Fletcher and White, 1973; Ourth, 1980). The complement is also present in the mucus, as well in the serum, regulating a series of interlinking enzyme reactions, leading to a cascade mechanism of disruption of cell membranes. C-reactive protein in serum can activate the complement and enhances phagocytosis. α-antiprotease found in normal rainbow trout serum (Ellis, 1981) is analogous to α_2-macroglobulin in mammals, another acute phase protein, and acts neutralizing the proteolytic activity of exo- and endotoxins by stabilizing macrophage lysosomes. Nonspecific cytotoxic leukocytes have been described in fish (Hinuma et al., 1980; Graves et al., 1984; Moody et al., 1985). Analogous to mammalian natural killer cells have been described in several bony fishes, lysing a variety of mammalian and teleosts cell lines, in particular during viral infections (Moody et al., 1985). Certain teleost species exhibit behavioural reactions compatible with type I hypersensitivity, whereas “mast cell-like” eosinophilic granular cells are involved in the degranulation of histamine granules (Ezeasor and Stokoe, 1980). Type II and type III hypersensitivity have not been documented in fish but probably occur.

1.4.6 Modulation of the immune response in fish

So far there are several ways to modulate the immune response in fish: temperature, neuroendocrine functions (stress enhances the production of cortisol), heavy metals (cadmium, lead, mercury, zinc), pollutants (dioxin, petroleum distillates), drugs (oxytetracycline), nutrition (ascorbic acid), species variability and pathogens (*Aeromonas salmonicida*, *Glugea stephani*).

2. MATERIALS AND METHODS

2.1 FISH

The specimens employed to carry out the different experiments have been obtained from FLI-Leibnitz Institute for Age Research– Jena (Germany). The production of the specimens followed this method:
the fish spawn the eggs on small glass vessels holding extra fine sand on the bottom. Every week the sand is filtered to isolate the eggs which are then stocked in Petri dishes filled with slightly wet peat and maintained in a at 28°-30°C for about three months. When ready to hatch (the eyes of the fries become visible through the shell), chilly soft water (16°-18°C) is added to the peat and within 24 hours the fries hatch. At first (first two weeks) the fries are fed several times a day on small brine shrimp naupli for about two weeks, then switch to frozen chironomid larvae. They grow rather rapidly reaching adult size in about 4-5 weeks. Adult fish are kept in 40 litres tanks in a centralized system located in two small fish rooms, up to 10 adult specimens per tank. The physic-chemical setting of the water was:

- pH 7.5
- 25°-27°C
- 300 µs/cm
- Nitrites and nitrates absent

Depending on the type of the experiment to carry out, the adult fish were fed twice a day with chironomid larvae. Caloric restriction groups were fed once a day, starting by 4weeks (or 11weeks in long lived strains as MZM3, MZM4/10 and MZM8/10) old, and sampled together with the oldest groups (control groups) in order to evaluate its effects in terms of lesion occurrence and marker expression.

2.2 SAMPLING

Depending on the experiment, the fish were sampled in a different way and with a different timing.

2.2.1 Evaluation of the immune system

Due to several different inconveniences, it's been possible only to analyse and compare young VS old fishes, and no one fish treated with resveratrol. We've been able to perform three tests, always utilizing the MZM4/10pl strain (with an expected lifespan of 20-21 weeks), sampled at 5 weeks (young fishes) and 18 weeks (old fishes).
The evaluation of the immune system along with the lifespan has been carried out by analysing the activity of macrophage cells. The cells have been collected from the cranial portion of the kidney and from the spleen, and tested for binding and phagocytosis activity as well as respiratory burst. Due to the small size of the specimens, the fishes were pooled up as five specimens per test for each group.

2.2.2 Histology, histochemistry and immunohistochemistry

Liver biopsies has been carried out in order to perform histological, histochemical and immunohistochemical evaluations. Two different sets of fish have been utilized, each one consisting of 6 groups, 10 to 20 fish per group:

Set #1
1) MZM-3 7weeks
2) MZM-3 14weeks (caloric restriction)
3) MZM-3 14weeks (control)
4) MZM-8/10t 14weeks
5) MZM-8/10p 14weeks
6) MZM-3 21weeks

with expected lifespan in MZM-3 > MZM-8/10t > MZM-8/10p

Set #2
1) GRZ 5weeks
2) GRZ 11weeks (caloric restriction)
3) GRZ 11weeks (control)
4) MZM-3 11weeks
5) MZM-3 21weeks (caloric restriction)
6) MZM-3 21weeks (control)
with expected lifespan in MZM-3 > GRZ

The different timing of sampling reflects the expected youth and oldness phases for each strain.

1st set	1) MZM-3 7weeks 2) MZM-3 14weeks CR (caloric restriction) 3) MZM-3 14weeks CTRL(control) 4) MZM-8/10t 14weeks 5) MZM-8/10p 14weeks 6) MZM-3 21weeks **Expected lifespan** **MZM-3 > MZM-8/10t > MZM-8/10p**	H&E, T.U.N.E.L., IHC (Bcl-2, p53, PCNA) **Statistical analysis** **T test (p<0,05)**
2nd set	1) GRZ 5weeks 2) GRZ 11weeks CR (caloric restriction) 3) GRZ 11weeks CTRL (control) 4) MZM-3 11weeks 5) MZM-3 21weeks CR (caloric restriction) 6) MZM-3 21weeks CTRL (control) **Expected lifespan** **MZM-3 > GRZ**	H&E, IHC (AGEs, RAGE) **Statistical analysis** **T test (p<0,05)**

2.3 COLLECTION OF DATA

2.3.1 Binding & phagocitosis assay

Five fishes per test were utilized for each trial. Here is the assay protocol, a modified method of Secombes (1990):

- Extract head kidney and spleen from the specimens and push then through a 100µm nylon mesh, using basic media [L-15 (Leibovitz medium; Invitrogen GIBCO, USA) + 1% antimicotic/antibiotic (Invitrogen GIBCO, USA) + 0,1% FBS (fetal bovine serum; Invitrogen GIBCO, USA)] to rinse
- Aspire the cell suspension and layer on a conical tube containing 2,5ml of 54% Percoll® (Sigma Chemicals, Germany) in PBS
- Spin for 15 min at 650g
- Remove interface and make up to an appropriate volume in order to count the cells using trypan blue solution, 0,4%(Sigma Chemicals, Germany) (1:1)
- Record counts of viable and non-viable cells
- Adjust viable cells concentration at $1x10^7$ cells/ml with basic media
- Seed 200µl of cell suspension on a positive charge slide at room T° for 60 min to allow the attachment of macrophage cells
- Wash the slides with PBS to wash off all non-adherent cells

- Add 200µl of inactivate yeast suspension (IYS; 1,2 x 10^8cells/ml), resuspended in colture medium and allow binding & phagocytosis for 90 min at room T°
- Wash the slides with PBS for three times
- Fix the slides by the air and dehydrate with rapid wash (30 sec) in 70% MeOH
- Air dry
- Stain the slides with MayGrünwald-Giemsa staining
- Count all the macrophage cells in the slides

2.3.2 Respiratory Burst assay

The same five fishes as for Binding & Phagocytosis assays were also utilized for Respiratory Burst assays. Here is the assay protocol, another modified method of Secombes (1990):

- Extract head kidney and spleen from the specimens and push then through a 100µm nylon mesh, using basic media [L-15 (Leibovitz medium; Invitrogen GIBCO, USA) + 1% antimicotic/antibiotic (Invitrogen GIBCO, USA) + 0,1% FBS (fetal bovine serum; Invitrogen GIBCO, USA)] to rinse
- Aspire the cell suspension and layer on a conical tube containing 2,5ml of 54% Percoll® (Sigma Chemicals, Germany) in PBS
- Spin for 15 min at 650g
- Remove interface and make up to an appropriate volume in order to count the cells using trypan blue solution, 0,4%(Sigma Chemicals, Germany) (1:1)
- Record counts of viable and non-viable cells
- Adjust viable cells concentration at $1x10^7$ cells/ml with basic media
- Plate out 100µl/well in a 96 wells Elisa plate, 3 to 5 wells for each of the below mentioned groups and 4 wells for counting nuclei
- Place at room T° for 60 min to allow the attachment of macrophage cells
- Remove media by dumping out the basic media and replace with 100µl/well of appropriate media as reported here below: a) Blank → basic media
  b) NBT → basic media + NBT (1mg/ml)
  c) N+P → basic media + NBT (1mg/ml) + PMA (5µg/ml)
  d) SOD → basic media + NBT (1mg/ml) + PMA (5µg/ml) + SOD (100UI/ml)

NBT (nitro-blue-tetrazolium, Sigma Chemicals, Germany)), PMA (phorbol 12-myristate 13-acetate, FLUKA Biochemika), SOD (superoxide dismutase, Sigma Chemicals, Germany)

The media of counting nuclei wells must be replace with basic media

- Incubate at room T° for 15 min
- Dump out the media
- Add 100µl/wells 70% MeOH, then wash with 70% MeOH two more times
- Air dry
- Add 120µl/wells KOH and 140µl/wells DMSO
- Mix/shake in microplate reader and read at 620-630nm

In order to normalize the number of cells utilized for the trial:

- Dump out media of counting cells wells
- Add 100µl/wells of lysis buffer
- Incubate for at least 5 min
- Using a 100µl pipetor, draw buffer up an down for 10 times while scraping the bottom with the pipet
- Remove suspension and count

2.3.3 Histology, in situ hybridization (T.U.N.E.L.) and immunohistochemistry

All the specimens underwent standard histological preparation:

- Fixation in 4% neutral buffered formaldehyde, 4% paraformaldehyde or Bouin's fixative solution
- Dehydration on crescent alcohol solutions
- Inclusion in paraffin
- Cut of 5 to 10 3µm thick slides

The slides underwent:

- routine histology staining (H&E) for histological evaluation
- reading at confocal microscope to evaluate fluorescent lipofuscins
- in situ hybridization with T.U.N.E.L. method to evaluate apoptosis rate (DeadEnd™ Fluorometric TUNEL System, Cat. # 7130, Promega Corporation, USA): procedures as indicated by manufactures
- immunohistochemistry for the following Ab:
  1. Bcl-2 to evaluate the attitude to tumorigenesis (pAb, Cat. # PC68T, Oncogene Research Products, USA). Dilution: 1:400
  2. p53 to evaluate DNA repairing activity (mAb [Pab240], sc-99, Santa Cruz Biotechnology, Inc., USA). Dilution: 1:100
  3. PCNA to evaluate parenchyma regeneration and cellular turnover (pAb [FL-261], sc-7907, Santa Cruz Biotechnology, Inc., USA). Dilution: 1:100
  4. AGE (pAb, AGE102-0.2M, Biologo, Germany). Dilution: 1:200
  5. RAGE (pAb, LS-C33936, Lifespan Biosciences, Germany). Dilution: 1:500

Tissue sections for all the above mentioned antibodies previously underwent epitope retrieval by heating in boiling water for 11minutes in a microwave oven while placed in citrate buffer, pH6,5. The section have successively treated with 3% hydrogen peroxide to block endogenous peroxydase activity. After overnight incubation in a moist chamber at 4°C with the primary antibodies, the antibody labelling was revealed with ABC-peroxydase (Vector Laboratories, USA). The enzymatic reaction was developed with 3-1-diaminobenzidine (DAB, Sigma Chemical, Germany) as chromogen substrate, by using Mayer's haematoxylin as nuclear counter-staining. As negative controls, the primary antibodies were substituted with TBS or non immune sera.
A 0-to-4 score has been assigned for each specimen undergoing H&E, confocal observation, AGEs and RAGE immunohistochemistry, due to the difficulty on assigning a specific score to either any different lesion or a cellular localization of positive reaction to fluorescent lipofuscins and AGEs/RAGE antibodies. In the case of morphological score, the result represents a semi-quantitative sum of the values obtained from steatosis and tumors analysis. About the remaining antibodies, 10 random fields at 40x magnification were used to count positive nuclei to the immunohistochemical reaction.

Steatosis	0	Normal
	1	Cellular swelling or diffused or localized light steatosis
	2	moderata diffused or localized moderate steatosis
	3	Severe steatosis
Tumors	0	no tumors
	1	Single Hepatoma or few small Hepatomas
	2	Several and well differentiated Hepatomas
	3	Small and diffused HCC
	4	Diffused HCC

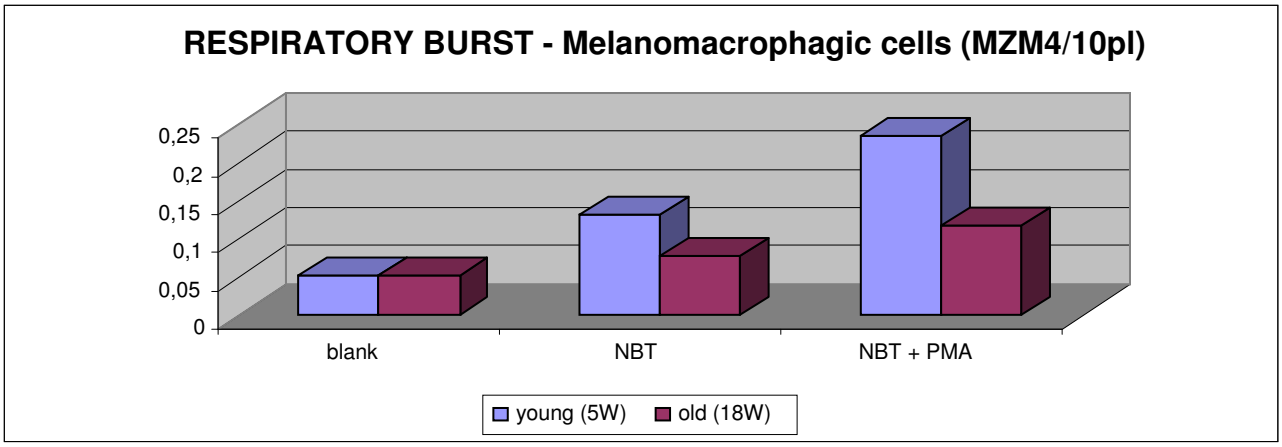
2.4 STATISTICAL ANALYSIS

All the data underwent Student T Test with $p<0,05$ at least for any result.

3. RESULTS

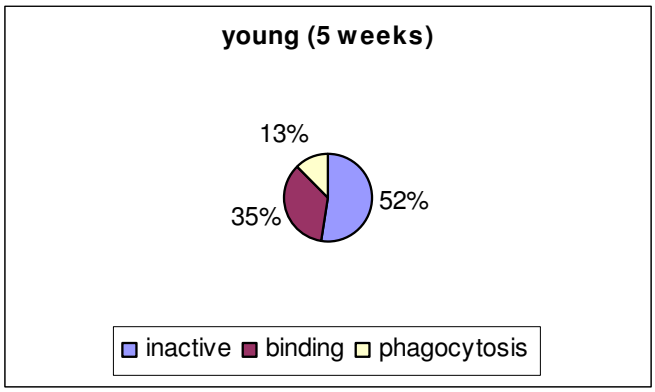
3.1 Respiratory burst

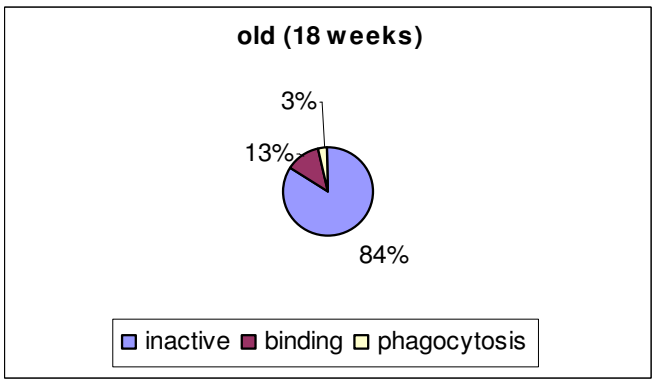
The graph shows a notable difference between young fish and old fish on the activity of the melanomacrophage cells as respiratory burst. This difference is even more evident in stimulated cells with PMA. All the sampled were also tested with SOD as positive control for the enzymatic reaction. ($p<0,05$)

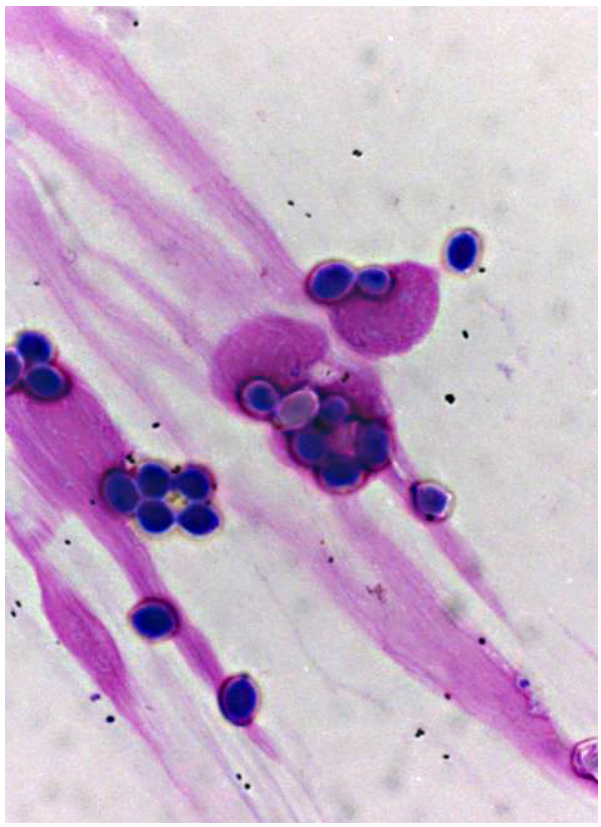


3.2 Binding & Phagocytosis

The images show a remarkable difference between young fishes and old fishes on the activity of the melanomacrophage cells as binding and phagocytosis of yeast spores. The figure on the right shows three active melanomacrophage cells. ($p<0,05$). MayGrünwald-Giemsa staining, 60X.

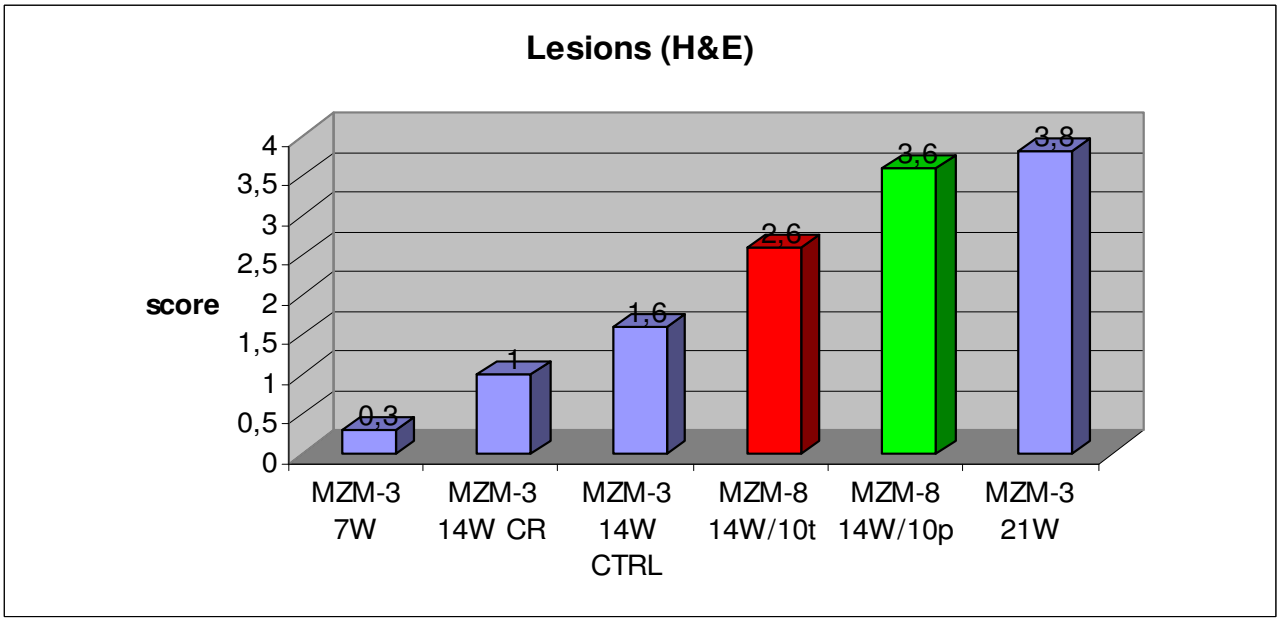




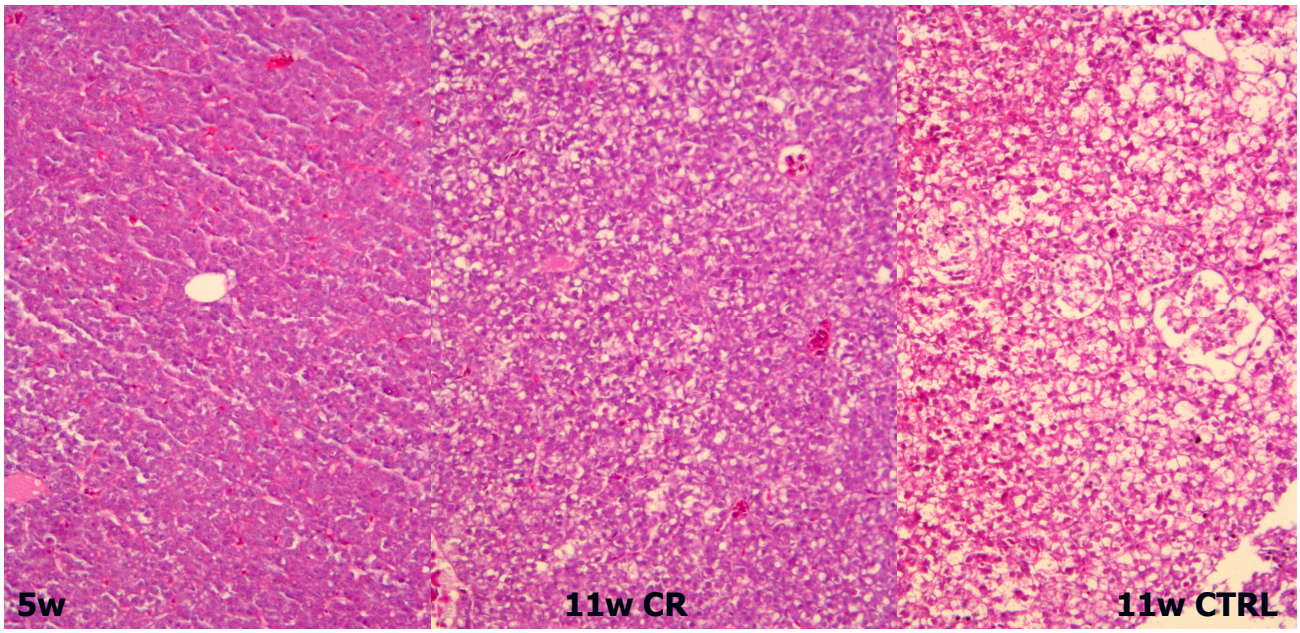


3.3 Histology and morphological alterations in liver

Both sets of fishes underwent standard histological evaluation (H&E) in order to observe morphological alterations of the liver. The graph regarding the first set of fishes displays an increasing recurrence of pathological alterations along with the age and the expected lifespan ($p<0,05$). The more relevant alterations reported were fatty degeneration up to steatosis and neoplastic nodules, classified as hepatomas or even hepatic cells carcinomas (HCC). Caloric restriction induced a significant decrease of pathological alterations. ($p<0,05$)

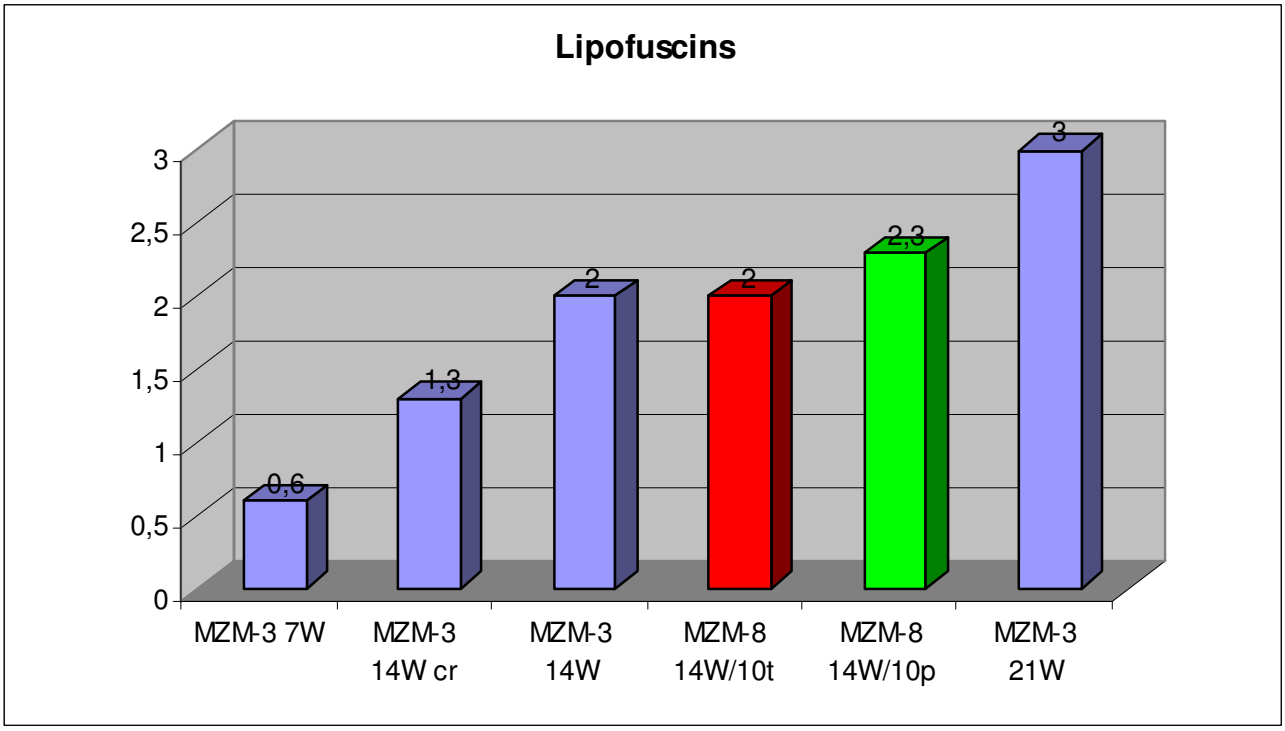


The figure shows the evolution of the degenerative modification (fatty degeneration) along with the age in *Nothobranchius furzeri*, GRZ strain. The 5weeks old specimen shows a healthy liver, while the older ones exhibit diffuse fatty degeneration, more severe in the control group wherein some neoplastic and pre-neoplastic nodules are even detectable. H&E, 10X.

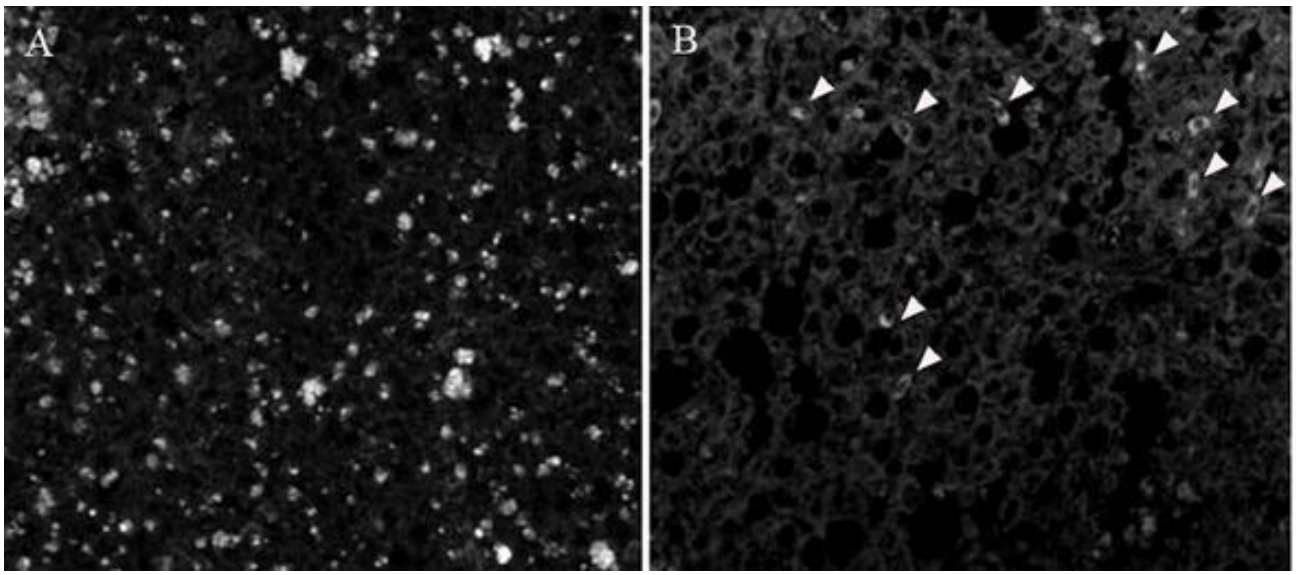


3.4 Lipofuscins

The graph shows a consistent increase in the accumulation of lipofuscins on the liver of *Nothobranchius furzeri* along with the age. The accumulation is significantly lower in the CR group, while the three different strain display no statistically significant variation each others. ($p<0,05$)

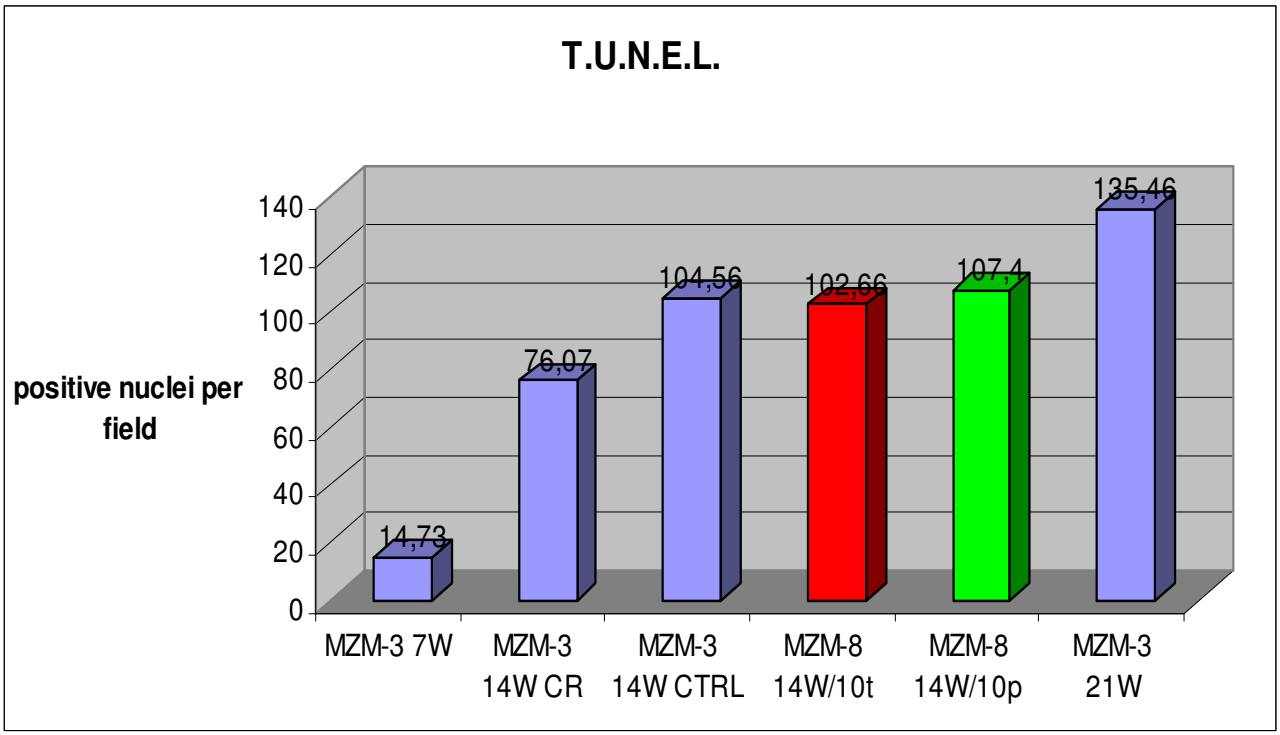


These two figures show the significant difference in autofluorescent lipofuscin accumulation between an old fish (MZM3 21 weeks; A) and a young fish (MZM3 7 weeks; B), small arrows. Confocal Microscope, 40X.

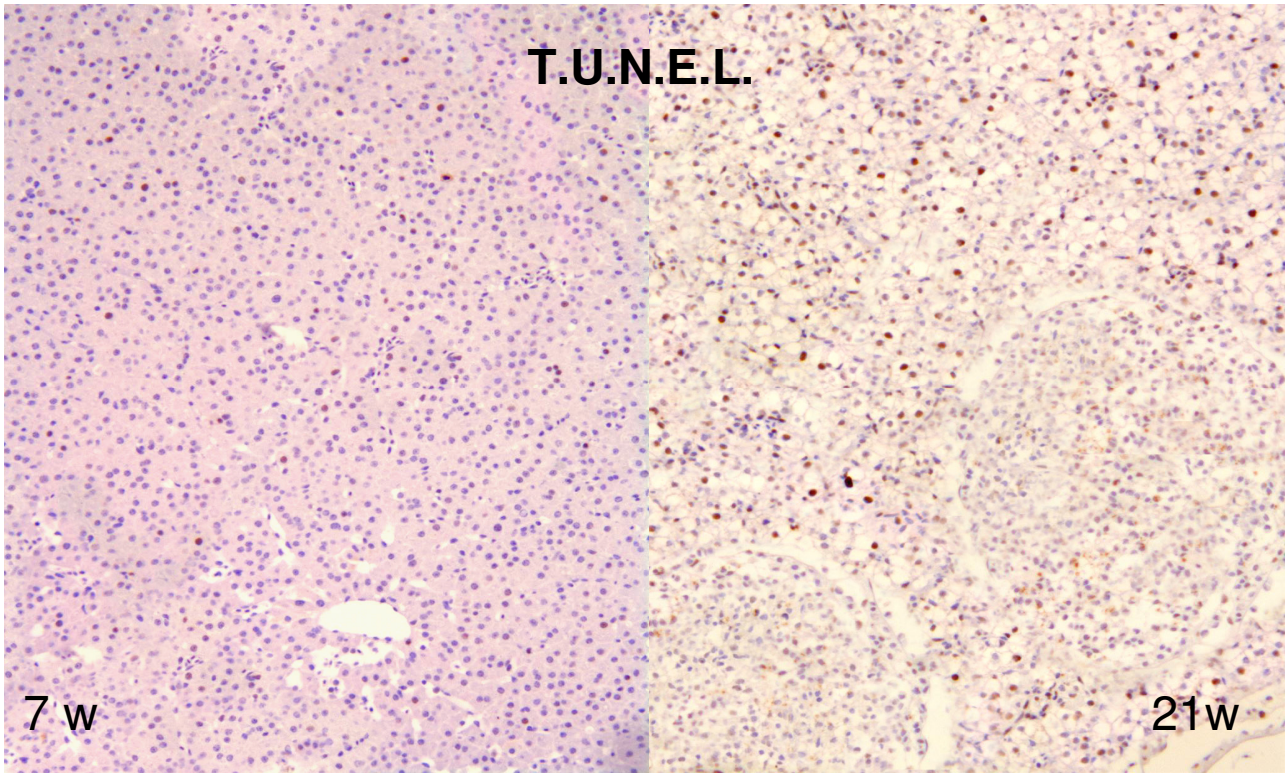


3.5 T.U.N.E.L.

The apoptosis rate grows up by the age but not by the strain. The CR group shows a lower apoptosis rate than CTRL group ($p<0,05$).

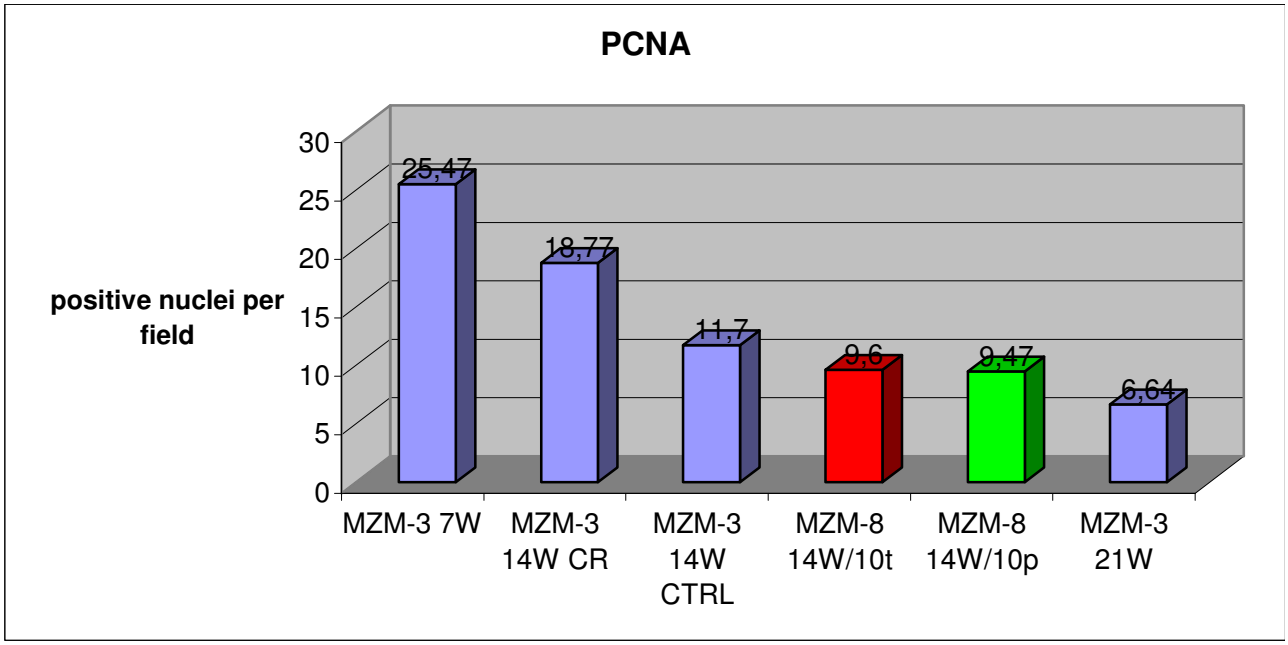


The figures display the distribution of the apoptotic nuclei: the positive nuclei were more numerous in old specimens and basically absent in neoplastic tissue. IHC, 10X

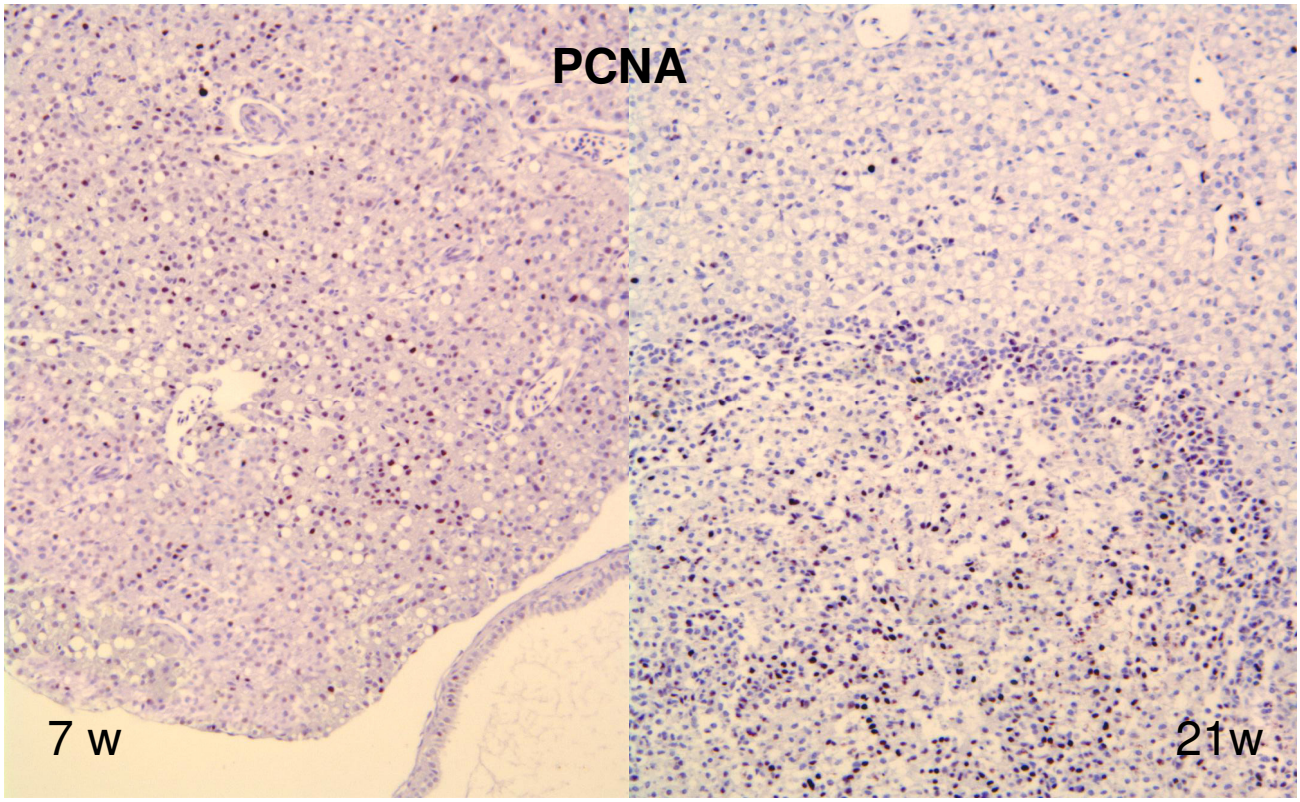


3.6 PCNA

The expression of PCNA tent to decrease according with the age and the strain. Moreover it look to be lower in CRTL group than CR group ($p<0,05$).

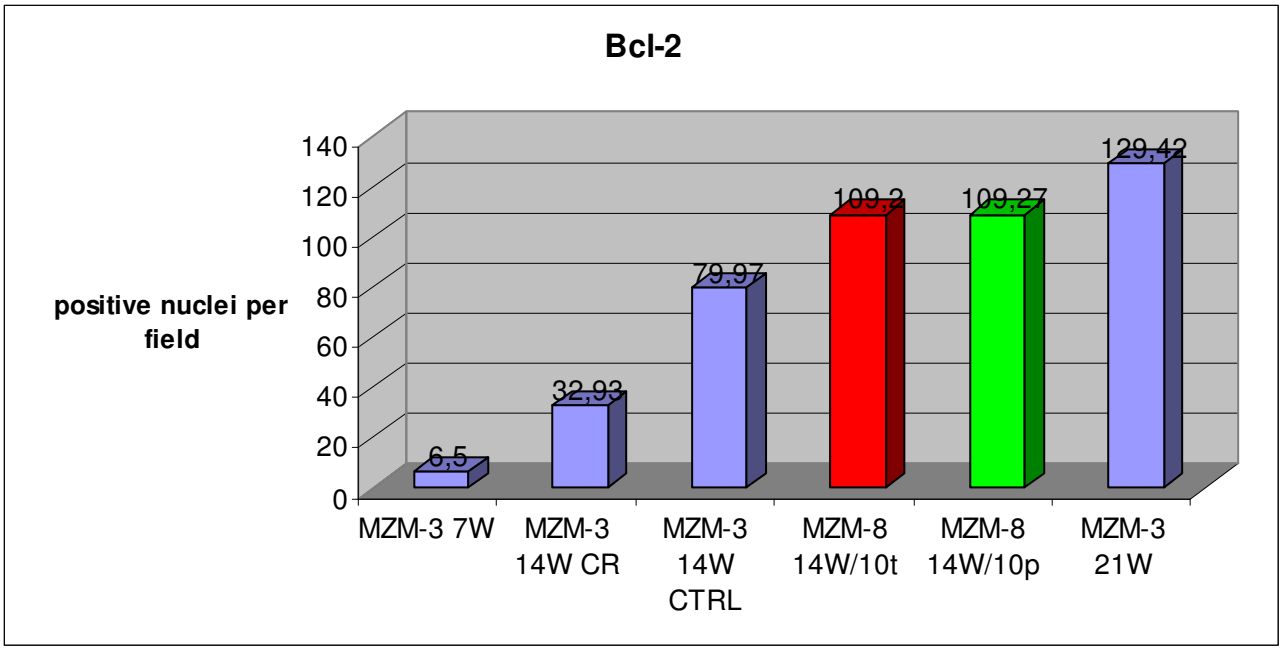


Neoplastic areas obviously shows a strong over-expression of the antigen and hence were not considered in the nuclei count. IHC, 10X

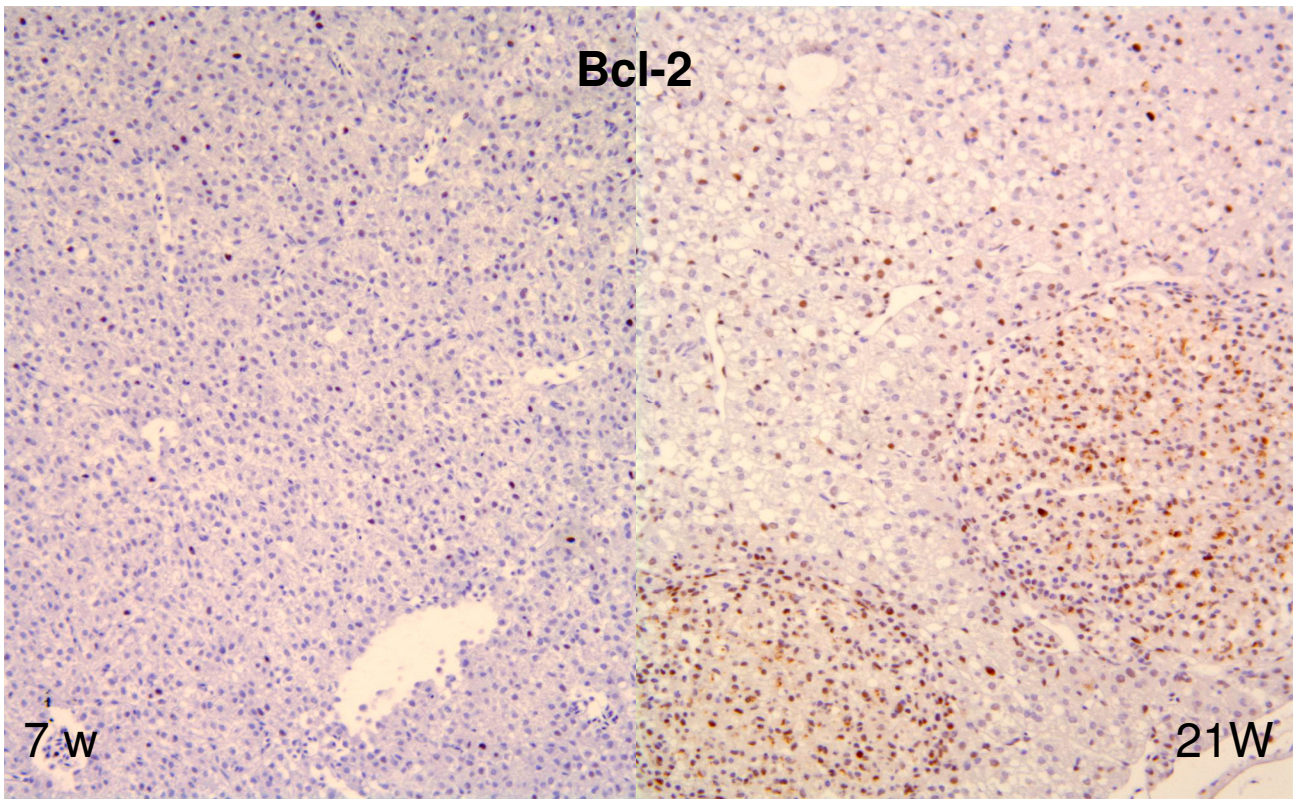


3.7 Bcl-2

Bcl-2 proteins expression grows according with the age and the strain. CR group shows a significantly lower expression than CTRL group ($p<0,05$).

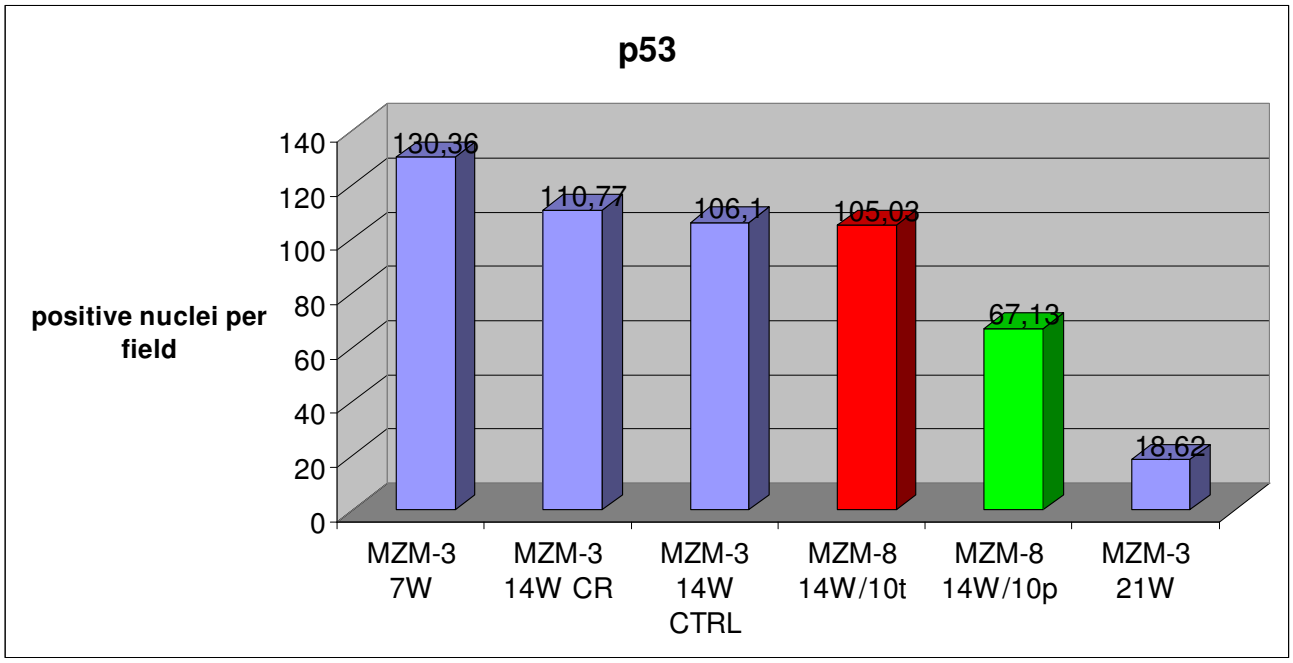


This family of proteins was over-expressed mostly on neoplastic portions as well as nearby areas and in massively degenerated/nearly-neoplastic areas. IHC, 10X

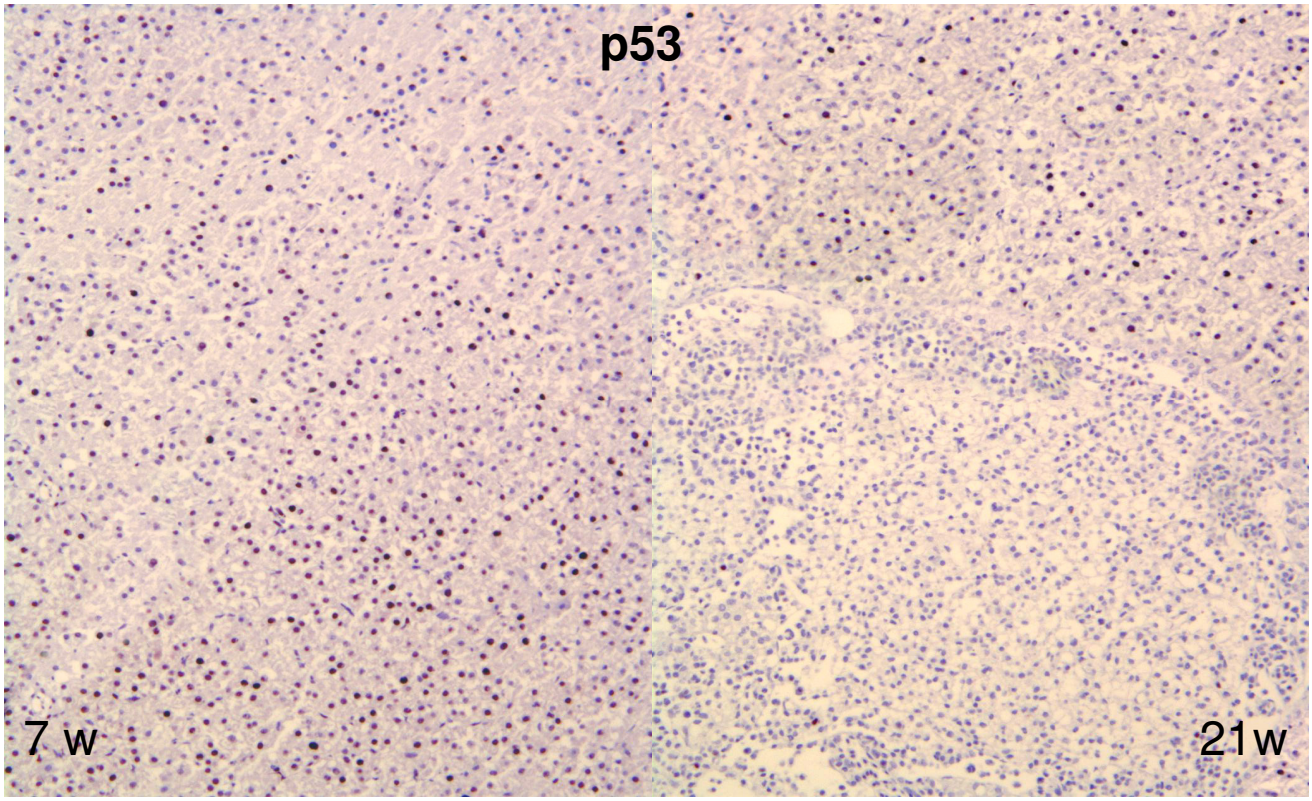


3.8 p53

A significant decrement of expression was reportable according with the age of sampling. Nevertheless p53 is the only parameter showing no differences between CR and CTRL groups. ($p<0,05$).



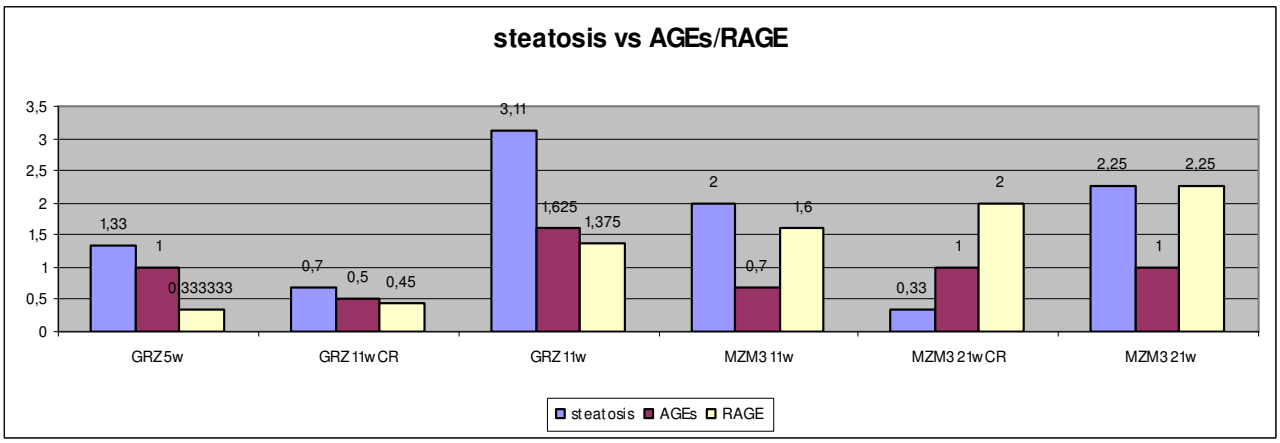
Neoplastic areas shows no expression. IHC, 10X

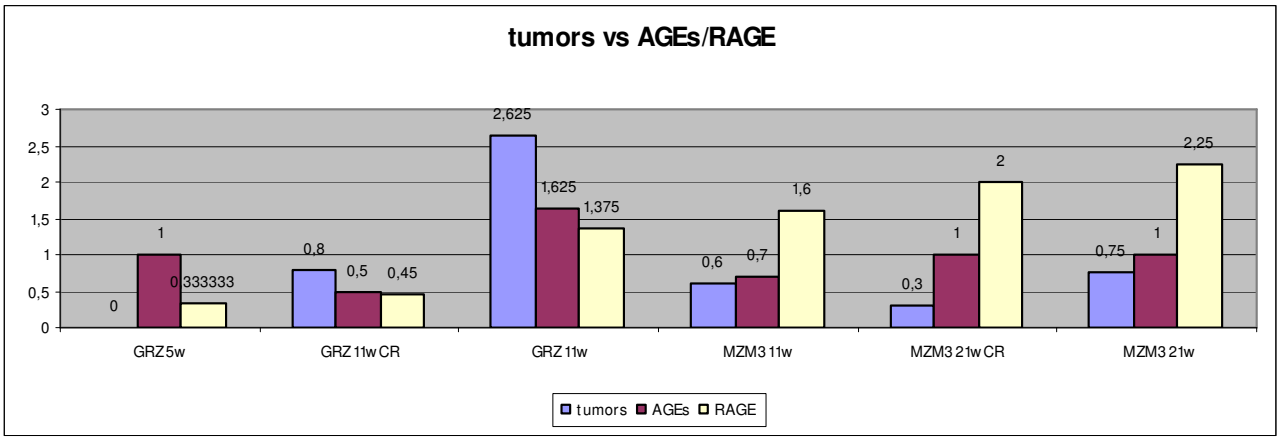


3.9 AGEs/RAGE

The first graph shows a nearly linear correlation between steatosis, AGEs and RAGE scores in GRZ strain, with an age-related increase of all the scores, although CR group displayed significantly lower scores than CTRL group. MZM3 group showed a different behaviour: the age-dependent enhancement of steatosis and RAGE scores was not accompanied by AGEs accumulation, which appeared slightly consistent in all the groups. However, CR group showed a steatosis and RAGE scores than CTRL group.

The second graph compares AGEs and RAGE distribution to the incidence and severity of neoplasms: GRZ strain showed a higher incidence of neoplasms associated with a strong expression of AGEs and RAGE. CR group showed a lower incidence of neoplasms than CTRL group as well as a lower AGE and RAGE expression. The moderately high score obtained by young GRZ specimens is due to a diffuse strong positivity of the membrane in degenerated hepatocytes, despite of the absence of neoplasms. In MZM3 strain, AGEs accumulation was fairly low and consistent in all the age classes when compared with RAGE expression, but looked to be directly correlated with neoplasm occurrence, which was consistently low. Neoplasm occurrence and severity was lower in CR group than in CTRL group.

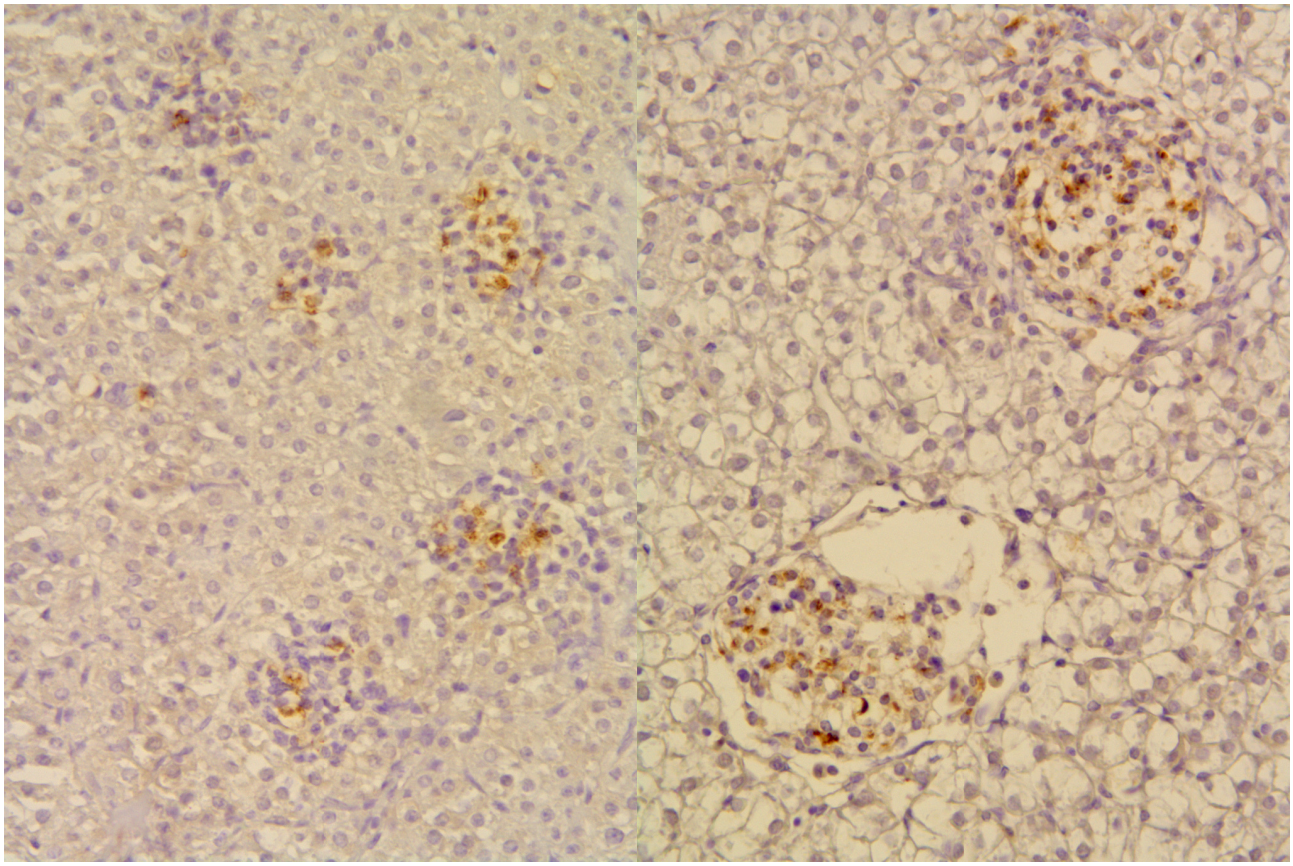




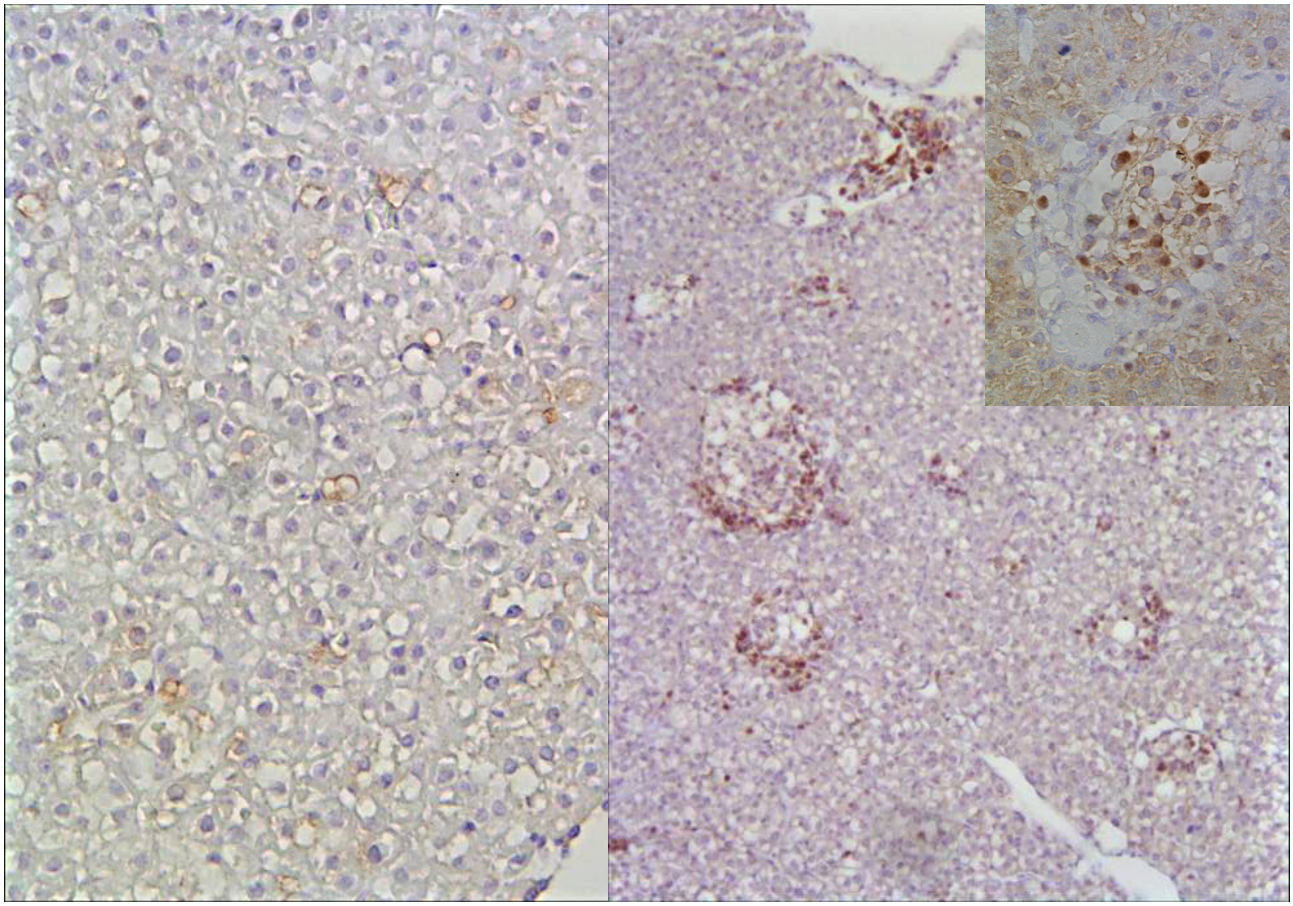
The accumulation of AGEs was primarily located in neoplastic cells, while RAGE were also over-expressed in pre-neoplastic cells and on the membrane of well-differentiated neoplastic cells and dystrophic/degenerated hepatocytes, particularly in young specimens.

	Localization	Staining (IHC)
AGEs	**Neoplastic cells**	**+++**
	Nearly-neoplastc cells	**++**
	Endothelial cells	**+++**
	Hepatic cells	**+**
RAGE	**Neoplastic cells**	**+++**
	Nearly-neoplastiche cells	**+++ (membrane)**
	Endothelial cells	**++**
	Hepatic cells	**++ (membrane)**

These two images show the primary sites of AGEs accumulation: both hepatomas (on the left) and hepatic cell carcinomas (HCC) displayed strong and selective specificity for AGEs accumulation, while the remaining portion of the liver was completely negative. IHC, 20X



These figures illustrate RAGE expression: in young specimens (7 weeks old GRZ specimens in particular, figure on the left), a strong positivity on the membrane of degenerated hepatocytes was recorded. Nevertheless, RAGE were also over-expressed on neoplastic cells , primarily in well differentiated neoplastic cells, which usually tent to maintain a peripheral position to the main neoplasm (figure on the right). On the insert, a small HCC characterized by a good positivity to RAGE in well and poorly-differentiated neoplastic cells. IHC, 20X (left), 10X (right) and 40X (insert)



4. DISCUSSION AND CONCLUSIONS

Nothobranchius furzeri is an emerging model for aging studies (Valenzano et al., 2006a; Valenzano et al., 2006b; Valenzano & Cellerino, 2006). This work presents the evaluation of the changes occurring on the immune system of this new model along the ageing as well as the pathological examination at histological and immunohistochemical level of the liver, a fundamental organ which regulate several essential physiological functions and overall the homeostasis of the organism. Finally, this was the first attempt to describe the molecular and physiopathological processes correlated with fast aging and spontaneous tumorigenesis in this species.

The first goal of this study was to compare the activity of the immune system between young and old specimens of *N. furzeri* strains in order to elucidate the reason for a comparison of time-depending lesions: in effect, the progressive decay of the immune response occurring along the lifespan has already been observed by Franceschi et al. (2006) in mammals: T lymphocytes tent to lessen their activity, while several immune organs (such as bone marrow, spleen and lymph nodes) get strongly reduced or even disappear. This situation may lead to an increasing susceptibility to infectious diseases as well as other physiological and immunological alterations and even neoplasms.

Considering the immune system of fish is characterized by a strong innate immunity supported by a weak acquired immunoresponse, we decide to evaluate the macrophage cells, as these cells are primarily involved in natural protection but also play an important role in the procession of the antigens and hence in the activation of the acquired immunity. This approach has been applied for the first time by Secombes (1990) in salmonids, then we modify these protocol to match our needs. The results we obtained from the comparison between young and old fish clearly confirmed the data coming out from the previous studies carried out in mammals: macrophage cells from old specimens exhibit a significantly lower respiratory burst, even when stimulated with PMA. Moreover, the same cells also showed a considerable reduction on motility and capability of bind and/or phagocyte foreign organisms. Although these finding don't clarify the insurgence of time-depending fatty degeneration on the liver, we might speculate the high incidence and severity of hepatic neoplasms in the oldest specimens is due to a failure of the immunological surveillance on new neoplastic cells.

To better support our finding, we planned in this study to add another group of old fish treated with resveratrol, in order to evaluate the efficiency of this molecule in retarding such degenerative or neoplastic alterations. Indeed there are some data coming from Valenzano et al. (2006b) demonstrating treated fish have a longer lifespan than CTRL specimens. An interesting finding of this study was also the fact that treated group showed at first a higher mortality rate than CTRL fish, but the survivals would have been surviving for a longer period. This finding might be explicate by another study carried out by Castro et al. (2008) where an important modulator effects of resveratrol on inflammatory responses in fish has been observed. Unfortunately we haven't had the opportunity to assess the efficiency of resveratrol as this group of specimens died during the experiment.

Although several organs displayed severe age-related lesions and several neoplasms (Cellerino, pers. comm..), the liver was chosen as target organ for our histological and immunohistochemical study, due to its high metabolic activity. Histological analysis revealed that, with increasing age, the severity of lesions and the incidence of neoplasms increased significantly. We can't still explain why old fish showed fatty degeneration up to strong steatosis, as this phenomenon is not reported or even observed in other *Nothobranchius* species. Since this fish was kept under lab conditions, a dietary reason was first proposed as cause of this degeneration, but the finding that 1) longer-lived *Nothobranchius* species fed on the same diet develop steatosis a later ages and 2) analogue lesions can be seen in wild captured fish (Cellerino, pers. comm.) suggest that steatosis is a natural age-related pathology in *N. furzeri* . Several interesting findings come out from morphological evaluation: 1) control groups showed a typical senile process, analogue to the one occurring in Mammals; 2) using different strains, we reported the same lesions and the same evolution of these

disorders being postponed according with different expected lifespan; 3) caloric restriction does affect the incidence of age-related diseases and spontaneous tumorigenesis.
The methods and antibody panel we used for our study confirmed these assumptions. In fact lipofuscins accumulation, defines usually as "the aging pigments", grow significantly along with the age, and CR considerably affected this process. An important finding is that the accumulation didn't vary among the different strains sampled at the same age. Analysing apoptosis rate, T.U.N.E.L. method stated the presence of ongoing aging process and, most importantly, the preserving effect of caloric restriction on hepatic tissue, while the different expected lifespan didn't affect the reaction. PCNA expression substantially confirmed these results, as it decreased according with age, but also with the strain, and was significantly affected by caloric restriction. We might speculate caloric restriction slowed down the metabolic activity of liver, preserving turn-over rate. PCNA was obviously over-expressed in neoplastic areas, which were not considered on this analysis. Indeed, to do so, we used Bcl-2 proteins: being this family of proteins a marker of neoplastic transformation, they were well expressed on old fish, and in any case in all the fish presenting neoplasms. Therefore it's not a coincidence that the highest values belonged to the oldest groups, and that CR group revealed a significant lower value than CTRL group. Both malign and benign cancers over-expressed this marker. P53 was the only parameter not affected by caloric restriction: moreover another short lifespan strain showed analogue results to MZM-3 groups at the same age. This suggests DNA damages might not be strictly linked with the occurrence of the hepatic disorders, even though its value decrease considerably along with aging.
Using the second set of fishes, we compared morphological results with AGEs accumulation and RAGE expression. For this study we could utilize GRZ strain, the shortest expected lifespan strain. AGEs is the acronym for Advanced Glycation End products, which were utilized to assess the scale of ongoing aging processes on this particular species. In effect, AGEs are a heterogeneous group of glycosylated proteins which accumulate during aging processes and play an important role in the pathogenesis of a variety of chronic diseases as well as in cancer genesis (Butscheid et al., 2007). AGEs produce an effect by binding with RAGE, a multiligand receptor of the immunoglobulin superfamily of cell surface molecules that binds molecules that have been irreversibly modified by non-enzymatic glycation and oxidation. Indeed RAGE also act as counter-receptors for HMGB1, S100/calgranuline and β-amiloid peptides. Interactions with these ligands activate important cell signalling pathways leading to the production of pro-inflammatory cytokines. RAGE are now considered to contribute to the progression of many chronic diseases (Yamamoto et al., 2001) and sepsis as well as in tumorigenesis by increasing tumour invasion and metastasis (Taguchi et al., 2000).
So far these molecules have been studied only on human and mouse and hence this study represents an important step forward. In our experiments the accumulation of AGEs was mainly located in neoplastic cells, while RAGE were also over-expressed in pre-neoplastic cells and on the membrane of well-differentiated neoplastic cells and dystrophic/degenerated hepatocytes, particularly in young specimens. This distribution deeply affected the comparison with morphological data. In fact the histological results obtained from the second set of fishes overlapped the ones achieved by the first set: aging processes affect the comparison of liver degeneration and neoplasm incidence. Moreover caloric restriction did influence the occurrence of age-related modifications and neoplasms in both GRZ and MZM-3 strains. When we split morphological scores and compared either steatosis or neoplasm scores with AGEs and RAGE, we obtained some interesting results: in GRZ strain both AGEs accumulation and RAGE expression grow together with degenerative lesions, while in MZM3 AGEs tent to a relatively low and consistent value, independently by the age, caloric restriction and RAGE expression. On the other hand, comparing neoplasm scores with AGEs and RAGE, there was a direct correlation between incidence and severity of neoplasm with AGEs accumulation in both strains, as in our trial MZM-3 strain developed consistently a low amount of tumours. We might explain such a different pattern of accumulation/expression analysing the activity of AGEs and RAGE. In effect, Hiwatashi et al. (2007) stated RAGE over-expression is also

correlated to hypoxic-degenerated condition of cells, as it happen in fatty degenerated hepatocytes. Moreover, RAGE is over-expressed in well-differentiated neoplastic cells, while poor-differentiated neoplastic cells loose it . This feature confirms and suggests its usage in prognostic evaluation of liver neoplasms (Kiyokazu et al. 2007). Conversely, a direct correlation between AGEs, primarily located in neoplastic areas, and tumour occurrence makes these molecules very interesting indicators of cancer genesis and evolution, therefore further studies are requested to figure out their exact role in these processes.

In conclusion we might assert ageing represents not only a physiological status, rather a real syndrome characterized by important modifications on the organism, affecting primary regulatory processes as the immunoresponse but also influencing the mechanisms of cell proliferation, the apoptotic pathways, the catabolites production and accumulation and even inducing the neoplastic transformation. Caloric restriction and resveratrol administration demonstrated to efficiently affect ageing processes by retarding the timing of comparison and reducing the severity of lesions and neoplasms insurgence in N. furzeri, but while the first method seemed to have no collateral effects, the latest still showed a residual “toxic” activity in the first phases of the administration: such an effect is still not clearly understood and more studies are required.

Finally, these results demonstrated the high feasibility of this fish as an excellent model to study the effects of aging processes and spontaneous cancer genesis. New data will be acquired with the completion of the DNA sequencing on this species so that new genes will be identify to promote or reduce ageing processes, and innovative pathways will be available to further studies.

5. REFERENCES

Agius C. (1985) The melano-macrophage centres of fish. Fish Immunology (Manning M.J. and Tatner M.F. eds.) Academic Press, London, 85-105

Agius C., RobertsR.J. (2003) Melano-macrophage centres and their role in fish pathology. J Fish Dis 26, 499-509

Barger J.L., Kayo T., Vann J.M., Arias E.B., Wang J., Hacker T.A., Wang Y., Raederstorff D., Morrow J.D., Leeuwenburgh C., Allison D.B., Saupe K.W., Cartee G.D., Weindruch R., Prolla T.A. (2008) A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS ONE 3(6), 2264

Benguria A., Kim S., Lai C.Y., Jazwinki S.M. (1999) Modulation of life-span by histone deacetylase genes in *Saccharomyces cerevisiae*. Mol Biol Cell 10(1o), 3125-3136

Butscheid M., Hauptvogel P., Fritz P., Klotz U., Alscher D.M. (2007) Hepatic expression of galectin-3 and receptor for advanced glycation end products in patients with liver disease. Journal of Clinical Pathology 60, 415-418

Colomba M., Gregorini A., Palma F. and Cristini C. (2005) Neurobiologia dell'invecchiamento – teorie biogenetiche dell'invecchiamento. La vecchiaia tra salute e malattia: aspetti biologici, psicologici e sociali (Cristini C., Rizzi R., Zago S..) Edizioni Pendragon

Diconza J.J. (1970) Some chacateristics of natural haemagglutinins founf in serum and mucus of catfish, Tachysurus australis. Aust J Exp Biol Med Sci 48, 515-523

Edelstein L.M. (1971) Melanin: a unique polymer. Pathobiology Annual (Ioachin H.L. ed.) Appleton-Century-Crofts, New York, 309

Ellis A.E. (1981) Non-specific defense mechanisms in fish and their role in diseases processes. Dev Biol Stand 49, 337-352

Evason K., Huang C., Yamben I., Covey D.F., Kornfeld K. (2005) Anticonvulsant medications extend worm life-span. Science 307 (5707), 258-262

Ezeasor D.N., Stokoe W.M. (1980) A cytochemical, light and electron microscope study of eosinophilic granule cells in the gut of rainbow trout *Salmo gairdneri* Richardson. J Fish Biol 17, 619-634

Finkel T. and Ilsa I. (2002) Surviving an aerobic environment: aging under oxidative stress. Geriatric times, vol III, issue 4

Fletcher T.C, White A. (1973) Lysozyme activity in the plaice (Pleuronetces platessa) Experimentia 29, 1283-1285

Franceschi C., Lio D., Caselli G., Caruso C., Candore G., Balisteri C.R., Listi F., Grimaldi M.P., Vasto S., Colonna-Romano G. (2006) Immunogenetics, gender and longevity. Ann N Y Acad Sci 1089, 516-537

Fuentealba D., Friguet B., Silva E. (2009) Advanced glycation end products induce photocrosslinking and oxidation of bovine lens proteins through type-I mechanism. Photochem Photobiol 85(1), 185-194

Genade T., Benedetti M., Terzibasi E., Roncaglia P., Valenzano D.R., Cattaneo A., Cellerino A. (2005) Annual fish of the genus Nothobranchius as a model system for aging research. Aging cell 4(5), 223-233

Gerhard G.S., Malek R., Murtha E.K., Cheng K. (2004) Zebrafish, Killifish, neither fish, both fish? J Gerontol A Biol Sci Med Sci 59, 873-875

Glenn J., Stitt A. (2009) The role of advanced glycation end products in retinal ageing and disease. Biochimica et biophysica acta 1790 (10), 1109–1116

Graves S.S, Evans D.L., Cobb D., Dawe D.L. (1984) Nonspecific cytotoxic cells in fish (*Ictalurus punctatus*) I. Optimum requirements for target cell lysis. Dev Comp Immunol 8, 293-302

Gugliucci A., Bendayan M. (1996) Renal fate of circulating advanced glycated end products (AGE): evidence for reabsorption and catabolism of AGE-peptides by renal proximal tubular cells. Diabetologia 39 (2), 149–160

Gul A., Rahman M.A., Hasnain S.N. (2009) Role of fructose concentration on cataractogenesis in senile diabetic and non-diabetic patients. Graefes Arch Clin Exp Ophthalmol 247(6), 809-814

Haus J., Carrithers J., Trappe S., Trappe T. (2007) Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle. J Appl Physiol (Bethesda, Md. : 1985) 103 (6), 2068–2076

Haynes L., Fuller L., McKinney E.C. (1988) Fc rereptors for shark IgG. Dev Comp Immunol 12, 561-571

Herrera M., Jagadeeswaran P. (2004) Annual fish as a genetic model for aging. J Gerontol A Biol Sci Med Sci 59 (2), 101-107

Hinuma S., Abo T., Kumagi K., Hata M. (1980) the potent activity of freshwater fish kidney cells in cell-killing I. Characterization and species-distribution of cytotoxicity. Dev Comp Immunol 4, 653-666

Hiwatashi K., Ueno S., Abeyama K., Kubo F., Sakoda M., Maruyama I., Hamanoue M., Natsugoe S., Aikou T. (2007) A novel function of the receptor foe Advanced Glycation End- products (RAGE) in association with tumorigenesis and tumor differentiation of HCC Ann Surg Onc 15 (3): 923-933

Kang H.L., Benzer S., Min K.T. (2002) Life extension in Drosophila by feeding a drug. Proc Natl Acad Sci U S A 99(2), 838-843

Kishi S., Uchiyama J., Baughman A.M., Goto T., Lin M.C., Tsai S.B. (2003) The zebrafish as a vertebrate model of functional aging and very gradual senescence. Exp Gerontol 38, 777-786

Koschinsky T., He C.J., Mitsuhashi T., Bucala R., Liu C., Buenting C., Heitmann K., Vlassara H. (1997) Orally absorbed reactive glycation products (glycotoxins): an environmental risk factor in diabetic nephropathy. Proc Natl Acad Sci (USA) 94 (12), 6474–6479

Labinskyy N., Mukhopadhyay P., Toth J., Szalai G., Veres M., Losonczy G., Pinto J.T., Pacher P., Ballabh P., Podlutsky A., Austad S.N., Csiszar A., Ungvari Z. (2009) Longevity is associated with increased vascular resistance to high glucose-induced oxidative stress and inflammatory gene expression in P. leucopus. Am J Physiol Heart Circ Physiol 296, 946-956

Liu R.K., Walford R.L. (1975) Mid-life temperature-tranfer effect on life-span on annual fish. J Gerontol 30(2), 129-131

Lobb C.J. (1987) Secretory immunity induced in catfish, *Ictalurus punctatus*, following bath immunization. Dev Comp Immunol 11, 727-738

Mainwaring G., Rowley A.F. (1985) Studies on granulocytes heterogeneity in helasmobranchs. Fish Immunology (Manning M.J. and Tatner M.F. eds.) Academic Press, London, pp.57-69

Malek R.L., Sajadi H., Abraham J., Grundy M.A., Gerhard G.S (2004) The effects of temperature reduction on gene expression and oxidative stress in muscle from adult zebrafish. Comp Biochem Physiol Toxicol Pharmacol 138(3), 363-73.

Marcato P.S., (2000) Anatomia e istologia patologica generale veterinaria. Soc. ed. Esculapio, Bologna

McClearn G.E. (1997) Heterogeneous reference populations in animal model researches in aging. ILAR J 38(3), 119-123

Migliaccio E., Giorgio M., Mele S., Pelicci G., Reboldi P., Pandolfi P.P., Lanfrancone L., Pelicci P.G. (1999) The p66shc adaptor protein controls oxidative stress response and life-span in mammals. Nature 402 (6759), 309-313

Miyata T., Oda O., Inagi R., Iida Y., Araki N., Yamada N., Horiuchi S., Taniguchi N., Maeda K., Kinoshita T. (1993) beta 2-Microglobulin modified with advanced glycation end products is a major component of hemodialysis-associated amyloidosis. J Clin Invest 92 (3), 1243–1252

Moody C.E., Serreze D.V., Reno P.W. (1985) Non-specific cytotoxic activity in teleost leukocytes. Dev Comp Immunol 9, 51-64

Ourth D.D. (1980) Secretory IgG, lysozyme and lymphocytes in the skin and mucus of the channel catfish, *Ictalurus punctatus*. Dev Comp Immunol 4, 65-74

Press C., Evensen o., Retain J.L., Landsverk T. (1996) Retention of furuncolosis vaccine components in Atlantic salmon, *Salmo salar* L., following different routes of administration. J Fish Dis 19, 215-224

Rudman D., Feller A., Schlenker A. (1990) Effects of human growth hormone in men over 60 years old. New Engl J Med 323 (1), 1-6

Secombes C.J., (1990) Isolation of salmonid macrophage and analysis of their killing activity. In Stolen J.S., Fletcher T.C., Anderson D.P., Kaattari S.L., RowleyA.F., editors. Techniques in Fish Immunology -1. Fair Haven, NJ: SOS Publications; (1990): 137-153

Semba R.D., Ferrucci, Sun, Beck, Dalal, Varadhan, Walston, Guralnik *et al*. (2009a). Advanced glycation end products and their circulating receptors predict cardiovascular disease mortality in older community-dwelling women. Aging Clin Exp Re 21 (2), 182–190

Semba R., Najjar S., Sun K., Lakatta E., Ferrucci L. (2009b) Serum carboxymethyl-lysine, an advanced glycation end product, is associated with increased aortic pulse wave velocity in adults. Am J Hypertens 22 (1), 74–79

Shaikh S., Nicholson L.F. (2008) Advanced glycation end products induce in vitro cross-linking of alpha-synuclein and accelerate the process of intracellular inclusion body formation. J Neurosci Res 86(9), 2071-2082

Simm A., Wagner J., Gursinsky T., Nass N., Friedrich I., Schinzel R., Czeslik E., Silber R.E., Scheubel R.J. (2007) Advanced glycation end products: a biomarker for age as an outcome predictor after cardiac surgery? Exp Gerontol 42(7), 668-675

Srikanth V., Maczurek A., Phan T., Steele M., Westcott B., Juskiw D., Münch G. (2009) Advanced glycation end products and their receptor RAGE in Alzheimer's disease. Neurobiol Aging 21

St. Louis-Cormier E.A., OsterlandC.K., Anderson P.D. (1984) Evidence of cutaneous secretory immune system in rainbow trout (*Salmo gardneri*). Dev Comp Immunol 8, 71-80

Taguchi A., Blood D.C., del Toro G. et al. (2000) Blockage of RAGE-amphoterin signalling suppresses tumour growth and metastases. Nature 405, 354-360

Tan K.C., Chow, Lam, Lam, Bucala, Betteridge, Ip (2006). "Advanced glycation endproducts in nondiabetic patients with obstructive sleep apnea". Sleep 29 (3), 329–333

Terzibasi E., Valenzano D.R., Benedetti M., Roncaglia P., Cattaneo A., Domenici L., Cellerino A. (2008) Large Differences in Aging Phenotype between Strains of the Short-Lived Annual Fish *Nothobranchius furzeri*. PLoS ONE 3(12): e3866. doi:10.1371/journal.pone.0003866

Tizard I. (1987) Veterinary Immunology. W.B. Saunders, Philadelphia

Valdesalici S., Cellerino, A. (2003) Extremely short lifespan in the annual fish Nothobranchius furzeri. Proceedings The Royal Society, London B, 189-191

Valenzano D.R., Terzibasi E., Genade T., Cattaneo A., Domenici L., Cellerino A. (2006a) Resveratrol Prolongs Lifespan and Retards the Onset of Age-Related Markers in a Short-Lived Vertebrate. Current Biology 16, 296–300

Valenzano D.R., Cellerino A. (2006b) Resveratrol and The Pharmacology of Aging. Cell Cycle 5, 10, e1-e6

Valenzano D.R., Terzibasi E., Cattaneo A., Domenici L., Cellerino A. (2006c) Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish *Nothobranchius furzeri*. Aging Cell 5, 275–278
Valenzano D.R., Terzibasi E., Cattaneo A., Domenici., Cellerino A. (2006d) Temperature affects longevity and age-related locomotor and cognitive decay in short lived fish Nothobranchius furzeri. Aging cell 5(3), 275-278

Wells-Knecht K.J., Zyzak D.V., Litchfield J.E., Thorpe S.R., Baynes J.W. (1995) Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. Biochemistry 34(11), 3702–3709

Wood W.B., Johnson T.E. (1982) Genetic analysis of the life-soan in Caenorhabditis elegans. Proc Natl Acad Sci U S A 79 (21), 6603-6607

Wood J.G., Rogina B., Lavu S., Hovitz K., Helfand S.L., Tatar M., Sinclair D. (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature 430, 686-689

Yamamoto Y., Kato I., Doi T., et al. (2001) Development and prevention of advanced diabetic nephropathy in RAGE-over-expressing mice. J Clin Invest 108, 261-268

Yan S.F., D'Agati, Schmidt, Ramasamy (2007a) Receptor for Advanced Glycation End products (RAGE): a formidable force in the pathogenesis of the cardiovascular complications of diabetes & aging. Curr Mol Med 7 (8), 699–710

Yan H.D., Li X.Z., Xie J.M., Li M. (2007b) Effects of advanced glycation end products on renal fibrosis and oxidative stress in cultured NRK-49F cells. Chin Med J 120 (9), 787–793.

Yen K., Mastitis J.W., Mobbs C.V. (2004) Life-span is not determined by metabolic rate: evidence from fish and C. elegans. Exp Gerontol 39 (6), 947-949

Zimmerman G.A., Meistrell M. 3rd, Bloom O., Cockroft K.M., Bianchi M., Risucci D., Broome J., Farmer P., Cerami A., Vlassara H., et al. (1995) Neurotoxicity of advanced glycation end products during focal stroke and neuroprotective effects of aminoguanidine. Proc Natl Acad Sci U S A 92(9), 3744-3748