Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
SCIENZE VETERINARIE

Ciclo XXX

Settore Concorsuale di afferenza: 07/H4

Settore Scientifico disciplinare: VET/07

Biomedical Research: Engineered C57BL/6 as Mouse Models for the Study of Treatments for Hutchinson-Gilford Progeria Syndrome (HGPS), and HER2-positive cancers.

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Esame finale anno 2018

To my special families

“ὁ δὲ ἀνεξέταστος βίος οὐ βιωτὸς ἀνθρώπῳ”
Ἀπολογία Σωκράτους, Πλάτων
“Life without enquiry is not worth living for a man”
Apology of Socrates, Plato

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Acknowledgements

I am sincerely grateful to the National Research Coucil, Institute of Molecular Genetics, Unit of Bologna, and in particular to Dr Lattanzi Giovanna, Dr Squarzoni Stefano, Dr Schena Elisa, Dr Capanni Cristina and Dr Loi Manuela. Thanks to the collaboration between our institutions we were able to conduct the researches described in Chapters 3-5 on Progeria Syndrome. Working together on this topic has been stimulating, always motivated and inspired by Sammi Basso's strength. I also thank the Anatomo-Patological Service of DIMEVET, and in particular Professor Sarli Giuseppe and Dr Pellegrino Valeria for their valuable contribution to the research, and the Service of Diagnostic for Images of DIMEVET, and in particular Professor Diana Alessia and Dr Linta Nikolina.

I am thankful to Professor Campadelli-Fiume for letting me be part of the interesting and innovative research on the treatment of HER2-positive cancers with oncolytic viruses and immunotherapy, described in Chapters 6 and 7. I would like to thank all the research group of the Department of Experimental, Diagnostic, and Speciality Medicine (DIMES), Alma Mater Studiorum – University of Bologna, and in particular thank you to Dr Gianni Tatiana and Dr Leoni Valerio.

A special thank you goes to Dr Barboni Catia; you taught me most of the things I now know. I am very pleased that I had the chance to work with Dr Sanapo Mara, Dr Casadio Chiara, Dr Santini Sara; all of you contributed to this research.

I am forever grateful to Dr Barbarossa Andrea, a colleague and a special friend. Thank you for the discussions, ideas, and feedbacks that have been absolutely invaluable.

Most of all, I thank my Tutor Professor Zaghini Anna, for guiding me and supporting me over these years. She has been an example of excellence as a mentor, instructor, and role model.

I want to express how thankful I am to thank my life companion Nico. He always supported me in my studies and carrier, teaching me that life is not always as complicated as I think, not if we challenge it together. You are my orange, big strong sun and the light of my life. Thank you to our little babies Mr Muffin, Apple Pie, Marsh and Mellow who are always there for me and teach me every day what pure and unconditioned love is.

Thanks to my amazing family for the love, support, and constant encouragement over the years. All my parents (more than two), my brothers (they are three), my wonderful and inspiring Zia Silvia and, oh, my Nonna Vale. Thank you to uncle Russel, who often helped me with the English and gave me a lot of valuable advice!

Finally, I would like to thank all my other families, my close friends. I can count on all of you and you never make me feel alone. In particular, thank you Jessica, Mabel, Sofia, Deborah, Giulia N, Giulia L, for your friendship since high school. Thanks to Jenny, Feffa, Chiari, Fede for all the support and great times since we started Uni.

List of abbreviations

53BP1	p53-binding protein 1
AIFA	Italian Medicines Agency
ANOVA	ANalysis of VAriance
Anti IL-6 R	Anti Interleukin-6 Receptor
ASCO	American Society of Clinical Oncology
ATP	Adenosine Triphosphate
ATR	Ataxia Telangiectasia Related
ATRA	All-Trans Retinoic Acid
BCS	Body Condition Score
BR	Biomedical Research
BRCA1	Breast Cancer Gene 1
BRD4	Bromodomain-Containing Protein 4
BSBT	Bioscienze Biotecnologie, DIMEVET
C	Faeces
C57BL/6	Black Six (mouse strain)
CAP	College of American Pathologists
CCAC	Canadian Council on Animal Care
CISH	Chromogenic *In Situ* Hybridization
CMT2B	Charcot-Marie-Tooth-Disease type 2B1
CNR	National Research Coucil, Institute of Molecular Genetics, Unit of Bologna
CR	Calorie Restriction
CRC	Colorectal Cancer
CTA	Clinical Trial Authorisation
CVD	Cardiovascular Disease
DAMP	Danger-Associated Molecular Pattern
DC	Dendritic cell
DIMES	Department of Experimental, Diagnostic and Specialty Medicine, Alma Mater Studiorum - University of Bologna, Italy
DIMEVET	Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna, Italy
DNA	Deoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
ECD	Glycosylated Extracellular Domain
ECHO-7	Enteric Cytopathic Human Orphan Type 7
ECM	Extracellular Matrix
EDMD	Emery-Dreyfus Muscular Dystrophy
EMA	European Medicine Agency
EMEA	European Agency for the Evaluation of Medicinal Products
ENU	N-ethyl-N-nitrosourea
ER	Endoplasmic Reticulum
ES cells	Embryonic Stem Cells
EURL-ECVAM	The European Union Reference Laboratory for Alternatives to Animal Testing
F	Freezing
FDA	Food and Drug Administration
Felasa	Federation for Laboratory Animal Science Associations
FISH	Fluorescence *In Situ* Hybridization
FPLD	Familial Partial Lipodystrophy of the Dunnigan Type
FT	Farmacology/Toxicology Unit, DIMEVET

FTIs	Farnesyltransferase Inhibitors
G	Grooming
GAGs	Glycosaminoglycans
GEMMs	Genetically Engineered Mouse Models
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
GSC	Germline Stem Cells
H&E	Hematoxylin and Eosin
HA	Hyaluronan
HDAC6	inhibitors of the histone deacetylase
HE	Humane Endpoints
HER	Human Epidermal Growth Factor Receptor
HGPS	Hutchinson-Gilford Progeria Syndrome
HMG-CoA	5-hydroxy-3-methylglutaryl-coenzyme A
HSV-1	Herpes Simplex Virus Type 1
HVEM	Herpesvirus Entry Mediator
ICH	International Conference on Harmonization
ICMT	Isoprenyl Cysteine O-Methyltransferase
IFN	Interferon
IHC	Immunohistochemistry
IHCP	Institute for Health and Consumer Protection
IL-12	Interleukin 12
IL-6	Interleukin 6
IMSR	International Mouse Strain Resource
IND	Investigational New Drug
INM	Inner Nuclear Membrane
IOR	Rizzoli Orthopaedic Institute
IP	Intraperitoneal
iPSCs	Induced Pluripotent Stem Cells
IQFISH	Fast Fluorescence *In Situ* Hybridization
IZSLER	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna
KI	Kyphosis Index
LGMD1B	Limb-Girdle Muscular Dystrophy Associated with Atrioventricular Conduction Disturbances
Lmna	Lamin A/C
$Lmna^{+/+}$	Wild type mice, used as control for $Lmna^{G609G}$ transgenic mice
$Lmna^{G609G/+}$	Heterozygous mice model for HGPS
$Lmna^{G609G/G609G}$	Homozygous mice model for HGPS
LON	Lonafarnib
MAA	Marketing Authorization Application
MAD	Mandibuloacral dysplasia
MBP	Myelin Basic Protein
MMTV	Mouse Mammary Tumour Virus
MS	Multiple Sclerosis
MSCs	Mesenchymal Stem Cells
MTD	Maximum Tolerated Dose
mTOR	Mammalian Target Of Rapamycin
NBF	Neutral Buffered Formalin
NCI	National Cancer Institute
NDA	New Drug Application
NET	Nuclear Envelope Transmembrane Proteins
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells

NIH	National Institute of Health
NK	Natural Killer
NLS	Nuclear Localization Signal
NOD	Non-Obese Diabetic (mice model)
NPC	Nuclear Pore Complexes
NRC	National Research Council
NSS	Numerical Scoring System
o-HSV	Oncolytic Herpes Simplex Virus Type 1
OFT	Open Field Test
ONM	Outer Nuclear Membrane
OV	Oncolytic Virus
PAMP	Pathogen-Associated Molecular Pattern
PAS	Periodic Acid-Schiff
PCR	Polymerase Chain Reaction
PD	Pharmacodynamics
PDL1	Programmed Cell Death 1 Ligand 1
PFU	Plaque Forming Unit
PI	Post Implantation
PI3K	Phosphatidyl Inositol 3 Kinase
PK	Pharmacokinetics
PPARɣ	Peroxisome Proliferator-Activated Receptor Gamma
PPE	Protective Personal Equipment
PPi	Inorganic Pyrophosphate
PRA	Pravastatin
PTEN	Phosphatase and Tensin Homolog
RAPA	Rapamycin
RD	Restrictive Dermopathy
RNA	Ribonucleid Acid
ROS	Radical Oxygen Species
SC	Subcutaneous
SCID	Severe Combined Immunodeficiency (mice model)
SEM	Standard Error of the Mean
SISH	Silver *In Situ* Hybridization
SOP	Standard Operating Procedure
STAL	Stabulario animali da laboratorio – DIMEVET
T-DM1	Ado-trastuzumab-emtansine
T-VEC	Talimogene Laherparepvecan
TBK1	TANK-binding kinase 1
TC	Time Spent in the Center
TF	Transcription Factors
TFU	Tumorsphere Forming Unit
TIC	Tumour-initiating Cells
TIL	Tumour infiltrating lymphocytes
TM	Time Spent Moving
TNF	Tumour Necrosis Factor
Trp53	Tumour Suppressor Gene
U	Urine
UD	Ulcerative Dermatitis
VDR	Vitamin D Receptor
VM	Vertical Movements
VSMCs	Vascular Smooth Muscle Cells
WAP	Whey Acidic Protein

WT	Wild Type
ZO	Zoledronic Acid

Abstract (English)

This thesis focuses on two engineered C57BL/6 mice models used to study treatments for Progera Syndrome and HER2-positive cancers. The first study, conducted in collaboration with the National Research Council, Institute of Molecular Genetics - Unit of Bologna, is on *Lmna*^{G609G} transgenic mice homologous for the genetic cause of Progeria Syndrome. The objective was to describe the model, giving information on the housing, breeding, welfare and progression of the disease. Similarities and differences between the model and human patients were highlighted and the acquired data will be essential in programming future studies using this animal model. Following this, a preliminary study was conducted on the mice, using a treatment of all-trans retinoic acid (0.4 mg/kg) combined with low doses of rapamycin (1 mg/kg) administered twice weekly intraperitoneally, in order to evaluate whether the mice's lifespan was improved. Results comparing treated and untreated groups were not significant. However, the animal groups were small in number and only males were considered, so future research will be needed. The second study reports the use of HER2 tolerant transgenic mice to investigate the efficacy of immunotherapy with oncolytic viruses. This study was performed in collaboration with the Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna. The mouse demonstrated to be a reliable model since it enabled the development of implanted HER2 cancer and allowed the virus replication within the tumour. Animals did not show any side effects and the safe profile of the virus was confirmed. To date we tested the efficacy of two viruses: R-LM113 and R-LM113-mIL-12. The latter gave better results, underlining the importance of the immune system in oncology and immune competent models in oncology research.

In conclusion, we can state that both animal models considered in this thesis have demonstrated their appropriateness for testing specific therapies.

Abstract (Italiano)

Questa tesi si focalizza su due modelli murini C57BL/6 geneticamente modificati per lo studio di terapie per la Sindrome Progerica e tumori HER2-positivi. La prima ricerca, in collaborazione con il Consiglio Nazionale delle Ricerche – Istituto di Genetica Molecolare di Bologna, studia il topo transgenico *Lmna*^{G609G} portatore della modificazione genetica che causa la Sindrome Progerica. L'obiettivo è stato di descrivere il modello, dando informazioni sull'allevamento, sul benessere e sulla progressione della malattia. Similitudini e differenze tra il modello animale e il paziente umano sono messe in evidenza e tutte le informazioni acquisite saranno utili per programmare studi futuri. Sul medesimo modello, è stato condotto uno studio preliminare per valutare l'efficacia del trattamento con una combinazione di acido retinoico (0.4 mg/kg) e rapamicina (1 mg/kg) somministrato intraperineo due volte a settimana nel prolungare l'aspettativa di vita. I risultati non sono stati significativi, ma data la preliminarietà dello studio saranno necessari ulteriori approfondimenti. La seconda ricerca riporta l'utilizzo di un topo transgenico reso tollerante al recettore HER2 (sovraespresso in alcuni tumori) per investigare l'efficacia di virus oncolitici in immunoterapia. Questo studio è stato condotto in collaborazione con il Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale dell'Università di Bologna. Il topo si è mostrato un modello affidabile in quanto è stato in grado di sviluppare masse tumorali dopo iniezione di cellule murine trasdotte con HER2 e ha permesso al virus di replicare. Gli animali non hanno mostrato effetti indesiderati e l'alto profilo di sicurezza virale è stato confermato. Ad oggi è stata valutata l'attività di due virus: R-LM113 and R-LM113-mIL-12. Il secondo ha dato migliori risultati, sottolineando l'importanza del sistema immunitario in oncologia e l'utilizzo di modelli animali immunocompetenti.
In conclusione, si può affermare che entrambi i modelli animali si sono rivelati appropriati per testare l'efficacia di terapie mirate.

Chapter 1

Introduction

Ideal animal models of human diseases should reproduce as much as possible the species and diseases that are to be investigated through the model. Genetically engineered animals have brought to remarkable advances in biomedical research. In particular, knock-in mice, in which a desired mutated DNA sequence is exchanged for the endogenous sequence, are considered workhorses in hypothesis-driven studies. On one hand, it is possible to induce genetic diseases that are typical of humans, study their mechanisms and pathways, and test the effect of therapies on such disease. On the other hand, it is possible to make animals tolerant to human receptors used for targeted therapies, for example in cancerology.

This thesis describes two researches conducted at the Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna. Both researches were conducted under the guidance of- and in collaboration with other research groups which are experts in the specific topics discussed below. In particular, the partnerships were with the National Research Council, Institute of Molecular Genetics - Unit of Bologna (Chapters 4-5), and the Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna (Chapter 7).

Chapter 2 defines the framework within all the thesis is done. This chapter discusses briefly on biomedical research, giving insights on the use of animals as models, regulations implicated with this kind of research and the role of veterinarians.

Chapter 3 describes many aspects of Progeria Syndrome in humans (HGPS) in order to better understand the following research in **Chapters 4** and **5**. In fact, **Chapter 4** describes the breeding, survival, growth, behaviour and phenotype of the transgenic *Lmna*^{G609G} knock-in mouse model for the human HGPS. This model was generated in 2011 by Professor C. López-Otín, University of Oviedo (Spain). The research described in this thesis gives additional information on the model, which was observed throughout a 2 years period. Phenotypic descriptions are very important when studying a disease through an animal model as discussed in **Chapter 2**. After a close and long study of the phenotype, similitudes and differences between the animal model and the HGPS patients are enlighten.

Afterwards, based on the characterization of the murine form of HGPS, the most recent knowledge of the aging process, and the results obtained from some *in vitro* tests recently

performed, the research continued with a preliminary evaluation of a treatment using the aforementioned mouse model. In **Chapter 5**, a combination of rapamycin (RAPA) and all-trans retinoic acid (ATRA), was studied in both the *Lmna*$^{G609G/G609G}$ and *Lmna*$^{G609G/+}$ models. RAPA-ATRA, already successfully studied *in vitro*, are potentially able to increase progerin degradation and decrease its expression and might improve pathological features *in vivo*.

The study reported in this thesis is only the beginning of a series of other preclinical studies that will be carried on. For example, on the basis of the results of **Chapter 5**, another preliminary study began testing higher doses of rapamycin. Also, we are testing the efficacy of an anti interleukin 6 receptor drug in ameliorating the mouse model's phenotype.

Chapter 6 briefly outlines topics that are useful to better understand the research described in **Chapter 7**. In particular, it outlines the importance of HER2 in oncotherapy, introduces to oncolytic virus-mediated immunotherapy and summarizes previous mouse models used to study HER2. HER2-positive cancers in cats and dogs are also briefly discussed in order to enlighten future possibilities using pets in clinical trials, simultaneously with humans. In fact, pets would benefit from innovative therapies and, at the same time, they would serve as additional models for humans.

Chapter 7 reports an *in vivo* preliminary research using transgenic mice tolerant for HER2 (HER2$^{+/-}$ C57BL/6) investigating the efficacy of immunotherapy with oncolytic viruses engineered at the Laboratory of Professor Campadelli-Fiume. Such preliminary research is meant to demonstrate the utility of HER2 transgenic mice as a model to study targeted therapies and in particular therapies involving the immune system. It also exemplifies why appropriate animal models are to date still useful in biomedical research, and it points out methods used to replace, refine and reduce animals in this experimentation.

Once again, a number of other experimental studies using HER2 transgenic mice as models are ongoing.

In **Chapter 8** final conclusions on all the topics discussed throughout the thesis are drawn out.

Chapter 2

Biomedical Research

2.1 What is Biomedical Research?

Biomedical research (BR) is a broad area of science that investigates and expands knowledge of biological processes and of causes of diseases through experimentation, observation, laboratory work, analysis, and testing. The aim of BR is to discover ways to prevent ill-health and to treat diseases and conditions that cause illness and death in both humans and animals. The contribution of many individuals with different backgrounds and skills, such as medical doctors, veterinarians, computer scientists, engineers, technicians, researchers, and a variety of scientists from different fields of the life sciences is essential for this kind of research.

A key component of this field of science is made by models and model systems. A biomedical model is a surrogate for a human being, or a human biologic system, that can be used to understand normal and abnormal function from gene to phenotype, and can also provide a basis for preventive or therapeutic intervention in human diseases (NRC, 1998).

We can broadly assemble the modern experimental models used in biology and medicine in two big categories: *in silico* methods (*dry lab*), based on the use of informatics and computer, and *biological methods* (*wet lab*), based on the use of purified molecules, cell cultures, perfused and isolated organs, and animal organisms, man included. The *wet lab*, in turn, is divided in *in vitro*, *ex vivo* and *in vivo* research (Garattini, 2007). Nowadays, we also talk of *moist lab* which sees both *dry* and *wet lab* staff working together in a multifunctional environment.

In vivo research (from Latin meaning “in the living”) refers to experimentation undergone in or on the living tissue of the whole body of a living organism. This kind of research differs from *ex vivo* (“out from the living”) and *in vitro* (“within glass”) research which are conducted outside the organism using bacteria, cell, tissue and organ cultures isolated from a living organisms and used directly or after they have been ‘immortalized’.

Biological systems are very complicated, it is then important to remember that only the use of the different experimental models in an integrated and complementary way can give useful

and global information. Each model has its own advantages and limitations, of which the researcher should be aware of.

Preclinical studies and clinical trials are two forms of *in vivo* research. The first, involve non-human animal models and is essential in order to discover more effective methods for diagnosing, treating, and curing diseases that affect both humans and animals. The latter, involve informed human volunteers, in order to measure the safety and effectiveness of drugs, procedures, or medical devices.

It is important to understand that basic research, applied research, *in vitro*, *ex vivo* and *in vivo* research all together represent building blocks of biomedical research. Rarely in biology and medicine it is possible to talk about “alternative methods”, and more often we need to think in a complementary way.

2.2 Overview of the Drug Development Process

A drug is a substance that, affecting the structure or function of the body, helps preventing, diagnosing or treating diseases. Before a drug can be placed on the market and prescribed to the animal or human population, it has to demonstrate that it is safe, active, effective and that it has a favourable risk-benefit ratio. It is easily understandable how behind prescription drugs there is a very challenging and complex process that can require many years of different types of studies, including preclinical ones and clinical trials.

Pharmacological research is regulated by the Food and Drug Administration (FDA) in the United States, by the European Medicines Agency (EMA, known as EMEA - European Agency for the Evaluation of Medicinal Products until 2004) in Europe, and, specifically in Italy, also by the Italian Medicines Agency (AIFA). Preclinical and clinical studies undergo the agencies evaluation in order to get the approval for placing the drug on the market. Drug development and preclinical trials are long and expensive processes with a high attrition rate. In fact, less than 10% of the compounds tested in clinical trials gain approval from the FDA (Zambrowicz & Sands, 2003; Sharpless & Depinho, 2006), which is, together with the european EMEA, the most demanding authority for drug approval in the world (Kashyap et al., 2013).

We will below take into consideration only, and briefly, *in vivo* preclinical and clinical studies. However, it is important to have in mind that these are only two of the numerous building blocks in biomedical research, and that the use of *in silico*, *in vitro* and *ex vivo* models give important preliminary data that need to be integrated and analysed together with data gained from *in vivo* models.

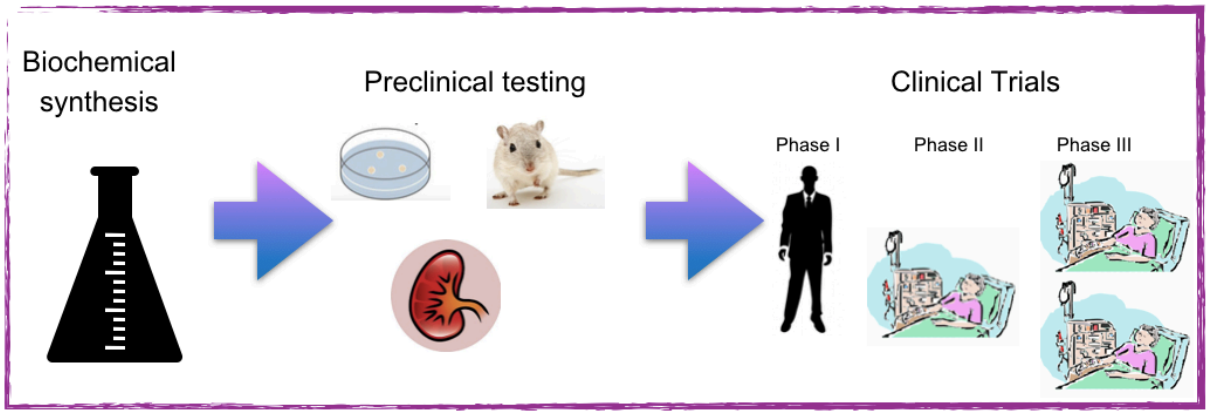


***Fig. 2.1**: Overview of the drug development process from biochemical synthesis to clinical trials.*

2.3 The Use of *In Vivo* Animal Models in Preclinical Studies

Preclinical investigations include evaluations of drug production and purity, and, more importantly, laboratory animal studies. Animal studies are especially useful to explore the drug's safety in doses equivalent to approximated human exposures, the pharmacokinetics (PK) (ie, drug absorption, distribution, metabolism, excretion, and potential drug–drug interactions), and pharmacodynamics (PD) (ie, mechanisms of action, and the relationship between drug levels and clinical response – known as PK/PD relationship). This data must be submitted for approval if the drug is to be further studied in human subjects (Umscheid et al., 2011), or more in general in the animal species to whom the drug is designed for.

In Europe, the use of animals for scientific purposes is regulated by the Directive 2010/63/EU *on the protection of animals used for scientific purposes* revising the Directive 86/609/EEC. This Directive was adopted on the 22nd of September 2010, and was received in Italy only in 2014 with the legislative decree n. 26/2014. This will be discussed in **Paragraph 2.6**.

Because animals are biologically similar to humans and are susceptible to many of the same diseases and health problems, researchers use animals as models during advanced stages of biomedical research. When animal models are employed in the study of human disease, they are frequently selected because of their similarity to humans in terms of genetics, anatomy, physiology, unlimited supply and ease of manipulation (Simmons, 2008).

A useful definition of 'animal model' is "a living organism in which normative biology or behaviour can be investigated, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal" (Held, 1983). Animal models are a pivotal component of preclinical biomedical research and will undoubtedly continue to do so, as their complexity and lifespan essentially mimics that of humans (Mitchell et al., 2015). Furthermore, to date still nothing can substitute for the complex functions of the whole living organism. Researchers avoid the use of animals whenever it is possible, and continue to look for other methods not involving them. However, unfortunately, animals are still necessary in this area because it is impractical, illegal, and unethical to use humans in early phases of research. Furthermore, just as a few examples, physiological parameters (such as blood pressure), behavioural studies, together with many other types of research, cannot be analysed if not in a complex *in vivo* model. There are many debates on predictability of these

models for human translation; however, often, the animal model is still the best approximation available.

Depending on the aim for what the animal models are used, they can be classified in exploratory, explanatory or predictive. Exploratory models are used to understand a biological mechanism (normal or abnormal), explanatory models to understand a biological problem, and predictive models to discover or quantify the impact of a treatment (Hau & Shapiro, 2011). The extent of resemblance of the biological structure in the animal with the corresponding structure in humans has been termed fidelity (Hau & Shapiro, 2011).

An animal model can also be considered homologous or isomorphic. The first, is one that shares the same cause of the disease with the target organism, the latter has a different disease cause but similar symptoms. However, often models rather be termed partial, not mimicking the entire human disease, even though they might be used to study certain aspects/treatments of the human disease (Hau & Shapiro, 2011).

In vivo models are crucial in the human disease setting for both aetiopathogenesis and therapy. The possibility to have animal experimental models that closely resemble the human situation enables researches to evaluate the effect of drugs in a more complete and significant way compared to a hypothetical cellular model.

Animal models of human disease can be divided in five big groups: spontaneous, induced, genetically modified, negative and orphan disease models (Hau & Shapiro, 2011). The first three are the most important ones.

Spontaneous models. These models, when referred to laboratory species, primarily consist in strains that are the result of the breeding of selected animals with spontaneous gene mutations. For example, NOD/LtJ mice are a natural polygenic model for autoimmune type 1 diabetes (Chaparro & DiLorenzo, 2010), nude and SCID mice reproduce different forms of immunodeficiency (Belizário, 2009), while MK/ReJ Nramp2$^{mk/+}$ mice mimic microcytic anaemia (Fleming et al., 1997). In a wider concept, also companion animals can have spontaneous diseases that closely resemble the ones in humans and therefore can be considered spontaneous models of human disease. For example, many myocardial disorders in cats and dogs, such as the arrhythmogenic right ventricular cardiomyopathies, can serve to increase the understanding of the genetic basis of human disease, including development of improved diagnostic assays and assessment of clinical therapies (Fox et al., 2007a). Despite the utility of

spontaneous mutations, these events are infrequent, occurring at a rate of ~5×10^{-6} per *locus* (Stanford et al., 2001).

Induced models, as the name implies, are healthy animals in which the condition to be investigated is experimentally induced. Early forward genetic approaches used radiation and chemical treatments to induce mutations (Doyle et al., 2012). For example, mutagenesis of mice using N-ethyl-N-nitrosourea (ENU), is still considered to be a very efficient method for obtaining mouse mutations in phenotype-driven screens (Salinger & Justice, 2008) and is still widely used in several national/international large-scale mutagenesis programs (for example: http://mutagenetix.utsouthwestern.edu/home.cfm). In fact, ENU produces random, single base-pair changes throughout the genome, and can produce a high frequency of mutant phenotypes (Carlson & Largaespada, 2005). Literature is also very rich of examples of diseases induced pharmacologically or through surgery, in which no genetic mutation is implied. Mouse surgical models led to pretty realistic brain and heart ischemic models used to validate pharmacological interventions, while advances in experimental surgery are nowadays very important to study therapeutic strategies using stem cells of different origin, especially using large animal models (Tarnavski, 2009; Harding et al., 2013). Also, pharmaceutical treatment with specific agents are able to reproduce human diseases. For example, the experimental autoimmune encephalomyelitis (EAE), obtained in the rat through injections of myelin basic protein (MBP), with all its weaknesses, is the most commonly used experimental model for the human inflammatory demyelinating disease, multiple sclerosis (MS). Many of the drugs that are in current or imminent use in MS have been developed, tested or validated on the basis of EAE studies (Constantinescu et al., 2011). However, few induced models completely mimic the aetiology, course, and the pathology of the target disease in the human (Hau & Shapiro, 2011).

Genetically engineered animals have many applications in biomedical research. These include understanding of gene function, modelling of human disease to either understand disease mechanisms or to aid drug development, and xenotransplantation. Genetic engineering and embryo technology manipulation, has brought to remarkable progresses in biomedical research. The Canadian Council on Animal Care (CCAC) gives to genetically engineered animals the following definition: “an animal that has had a change in its nuclear or mitochondrial DNA (addition, deletion, or substitution of some part of the animal’s genetic material or insertion of foreign DNA) achieved through a deliberate human technological intervention” (Ormandy

et al., 2011). However, this definition is broad, including those animals that have undergone induced mutations (by chemicals or radiations) and those that have been cloned. Transgenic animals, created through the addition, removal, or alteration of genes can represent quite remarkable and significant disease models (Hau & Shapiro, 2011). In this contest, mice are the first specie chosen for experiments (Hau & Shapiro, 2011). Nevertheless, researchers use technology to generate also transgenic rats (Filipiak & Saunders, 2006; Geurts et al., 2009) cats (Wongsrikeao et al., 2011), dogs (Hong et al., 2009), rabbits, pigs, sheep (Hammer et al., 1985; Nohmi et al., 2017; Sper et al., 2017), goats, cattle, chickens (Wolf et al., 2000), zebrafish (Higashijima et al., 2000), and non-human primates (Sasaki et al., 2009), just to name a few (Ormandy et al., 2011). However, not always genetically engineered animal models reflect accurately the human condition, so the limitations of such models should always be taken into consideration (Wells, 2010).

Negative models are species, strains or breeds in which a certain disease doesn't develop. Their main application is in studies on the mechanisms of resistance that seek to gain insight into its physiological basis (Hau & Shapiro, 2011).

Finally, an orphan model is a non-human species that has a naturally functional disorder that has not been yet described in humans, and that is recognized when a similar human disease is later identified. In this case, the literature generated in veterinary medicine may be useful when humans are discovered to suffer from a similar disease (Hau & Shapiro, 2011).

The ideal model may not exist but it is important to seek for the most appropriate one. The meaning of this statement (i.e., appropriate model) is well described by Held (1983). The author premises that the animal model must be relevant, in other words it must be comparable to a phenomenon in the species we attempt to investigate through the model. Therefore, we need to consider the appropriate species and the appropriate level of microbiologic and genetic definition. First of all, the model should accurately reproduce the disease or lesion under study. Second, it should be available to multiple investigators and easy to export. Third, the species should be polytocous – producing multiple young at each birth. Fourth, the animal should be of a size that permits multiple biopsies of samples. Fifth, the model should be available in multiple species. Sixth, it should survive long enough to be usable. Finally, it should fit into facilities of most laboratories and it should be easy to handle by most investigators. These last criterions are understandable and reasonable; however, convenience should not be the determining factor in the selection of the model (Held, 1983).

Animal models have contributed greatly to our knowledge of a multitude of different biological processes (Mitchell et al., 2015) and have greatly improved the understanding of the cause and progression of human genetic diseases. Furthermore, they have proven to be a useful tool for discovering targets for therapeutic drugs. However, despite some treatments resulted promising in some animal models, these do not always translate to human clinical trials, especially in oncology. Most available animal models are made in mice, and they recreate some aspects of the particular disease. However, few, if any, replicate all the symptoms (Simmons, 2008). Ideally, more than one animal model should be used to represent the diversity seen in most human disorders.

Independently from what the future holds for animal models, it is important to promote the sharing of resources, knowledge and effort toward the common goal of improving the health and well-being of all species and is providing to be a powerful adjunct to traditional laboratory animal models in a “one medicine” concept (Zinsstag et al., 2011).

2.4 Mice as Animal Models

Rodents are the most common type of mammal employed in experimental studies. Among them, mice (*Mus musculus*) definitely hold the record.

Human and mice probably share the most longer relationship between two mammals, even though their evolutionary lineages diverged more than 96 million years ago (Nei et al., 2001). It is interesting to note that in the last 10,000 years mice have been living side to side with humans, taking advantage of human food supplies and shelters, and have been a source of several human diseases (Doyle et al., 2012). Nonetheless, in the last 30 years also thanks to the new genetic technologies, mice have been our greatest ally in life science discoveries.

For many years, the inbreeding of mouse strains has evolved into a genetic tool that has been essential for mapping and identifying genes with important implications for human biology and disease (Nguyen & Xu, 2008). Compared to other genetically tractable organism, *Mus musculus* offers a close insight of humankind in terms of similarity in the underlying tissue structure and organization, and especially physiology (Nguyen & Xu, 2008). In 2002, the genome of C57BL/6J, a very common used mouse strain in biomedical research, was sequenced for the first time, and it was no surprize when it was discovered that of ~30,000 genes in both mice and humans approximately 99% are homologue (Mouse Genome Sequencing Consortium, 2002).

Genetically defined mouse models, including inbred, recombinant inbred, consomic, congenic, outbred, and genetically modified strains, have now become easily available to research institutions through the world (International Mouse Strain Resource – IMSR).

Currently, using the existing mouse strains (more than 24,000) and the genetically modified mouse embryonic stem cells (more than 209,000) that have been entered into the IMSR, there is the potential to generate over 200,000 mutant mouse strains (Fahey et al., 2013). The number of genetically defined mouse models is increasing and this trend is likely to continue as models are developed to answer questions about specific gene functions and gene–gene interactions (Fahey et al., 2013).

As previously described, early studies of mouse genetics were based on breeding mice with selective spontaneous mutations. A second step was represented by induced mice mutations with chemicals or radiations. In addition to these early mutagenic approaches, other strategies have been developed and employed based on directed gene recombination events, such as “gene trapping” approaches and insertional mutagenesis (Doyle et al., 2012).

Genetically engineered mouse models (GEMMs) are widely used and have proven to be a powerful tool in the drug discovery processes (van der Weyden et al., 2011; Ericsson et al., 2013; Lee, 2014), revolutionizing how we query gene function (Nguyen & Xu, 2008). "Gene trapping" is a strategy that relies on engineered recombination events to identify genes with provocative patterns of expression through the use of reporter genes such as beta-galactosidase or Green Fluorescent Protein (GFP). In contrast, random integration mutants have been described based on retroviral DNA insertion in the mouse genome, recombination-mediated events using transposons such as *Sleeping Beauty* and *PiggyBac*, and mutants resulting from DNA integration events associated with the production of transgenic mice (Doyle et al., 2012). Gene transfer technologies have allowed for novel genetic alterations approaching a specific gene of interest. Specifically, the abilities to introduce exogenous structural and regulatory gene sequences into the mouse genome (i.e., transgenic mice) and/or to ablate/modify the expression of specific endogenous mouse genes (i.e., knock-out/knock-in mice) have alone propelled this animal to the forefront of biomedical research of human health and disease pathology (Doyle et al., 2012). The concept of genetically engineering a mouse is very simple: devise a specific genetic modification in a chromosome of embryonic stem cells (ES cells) and use these modified cells to create mice that can transmit the new feature to their offspring. The method's straightforwardness rests on two principles: the ability to exchange specific chromosomal DNA sequences in mammalian cells by means of homologous recombination and the manipulation of embryonic stem cells in a way that allows inheritance of the genetic modification (**Fig. 2.2**) (Manis, 2007).

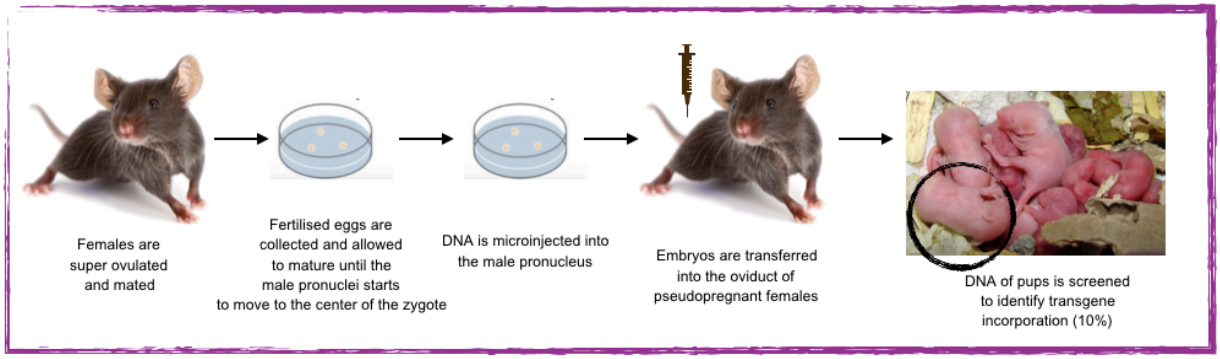


Fig. 2.2: *Overview of the creation of transgenic mice.*
DNA introduction into a pronucleus of a fertilized egg; random DNA integration into a single chromosomal location; injection of the eggs into a surrogate mother; the surrogate completes the gestation and produces mice with the transgene (only 2% of the injected eggs survive) (Adapted from Doyle et al., 2012).

Initially, a method was developed by which a specific gene in an ES cell could be inactivated. The genetically altered cell, after implantation into a surrogate mother, ultimately gives rise to a strain of mice that is homozygous for the inert gene — the "knock-out mouse" (**Fig. 2.3**). This allows to use experimentally targeted mutations to test a gene's functional role prospectively, differently from relying on spontaneous mutations. The generation of knock-out mice lacking the Trp53 tumour suppressor gene ($p53^{-/-}$ mice) is a classic example of the usefulness of this technology to model human carcinogenesis (Donehower et al., 1992).

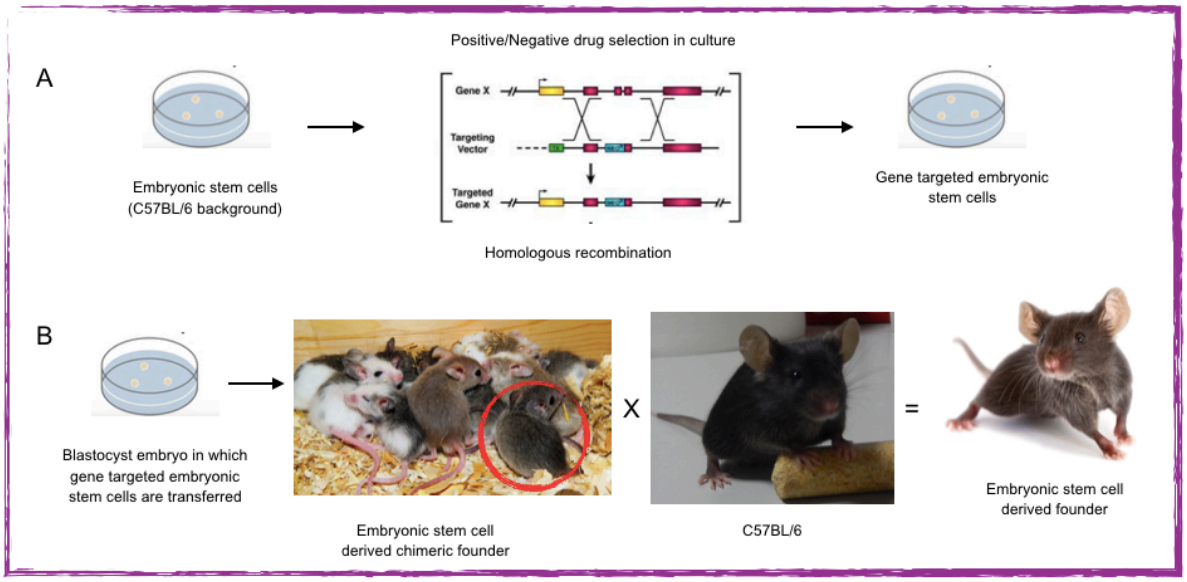


Fig. 2.3: *Production of knock-out mice.*
A) *ES cells are transfected with DNA targeting construct containing specific regions of homology to the gene of interest; homologous recombination events occur leading to a 1-to-1 replacement of the endogenous gene with sequence derived from the targeting construct.* ***B)*** *Targeted ES cell clones containing the appropriate genetic changes that ablate expression of the gene of interest are transferred to the blastocoel cavities of 3.5 days blastocyst embryos and, in turn, the embryos are transferred to surrogate mothers where gestation is completed. ES cell-derived chimeric founder mice with varying degrees of chimerism are generated. Commonly, one or more high chimeric founders are backcrossed with mice of the background strain of interest (Adapted from Doyle et al., 2012).*

Then, the "knock-in" method was developed, in which a mutated DNA sequence is exchanged for the endogenous sequence without any other disruption of the gene. With this method, it is possible to replace a gene sequence with a sequence of the investigator's choice and to delete unnecessary sequences (Manis, 2007) (**Fig. 2.4**).

Knock-in mice arose from a more sophisticated use of existing strategies employed in earlier gene targeting efforts rather than the development of new technologies/methods (Doyle et al., 2012). There are considered to be the "next-generation workhorses" of hypothesis-driven

studies whose goals are to define gene function and to integrate these definitions into broader models with which to explain organismal biochemistry and physiology (Doyle et al., 2012).

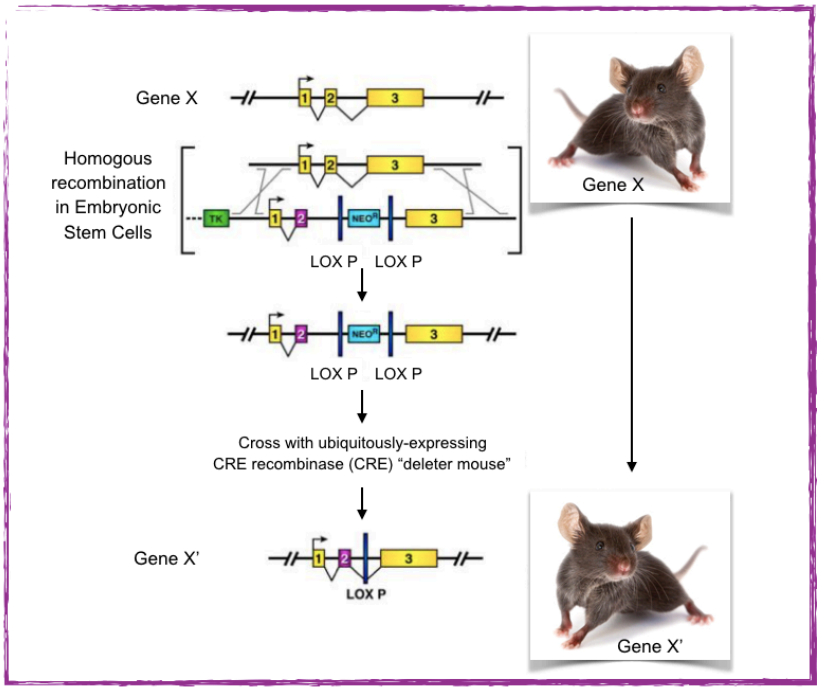


***Fig. 2.4**: Production of knock-in mice.*
Gene knock-in mice similar to their earlier knockout counterparts are generated in the two-staged process described above. The DNA targeting construct not only contains specific regions of gene homology but also has a uniquely engineered mutation or sequence change such that the 1-to-1 replacement of the endogenous gene with sequence derived from the targeting construct following transfection into ES cells yields an allele in the genome of these cells containing this new sequence variant (Adapted from Doyle et al., 2012).

Theoretically, mutant mice for every predicted gene can be generated through the ES cells approach. However, this procedure remains technically challenging, expensive and time-consuming. Consequently, investigators are converting only selected individual ES cell lines in which there are indications that the mutated gene may be involved in the process of interest (Nguyen & Xu, 2008).

In addition to ES cells, the germline stem cells (GSC) isolated from the neonatal mouse testis can also be genetically modified and transmitted upon injection into the testis (Kanatsu-Shinohara et al., 2004). It is also possible that a population of GSC with different genetic alterations can be injected into a single male to produce multiple mutant progeny (Nguyen & Xu, 2008). Finally, somatic cloning provides another novel way to produce genetically modified animals (Aoi et al., 2008; Hanna et al., 2008).

The use of the mouse is likely to be widened by humanizing the mouse, in other words this means that human tissues and cells or genes are reconstituted in the mouse. These mice

provide experimental models for directly studying the activities of human genes and their gene products and for developing and testing therapeutics against the human gene products (Nguyen & Xu, 2008). Mice with human “immune systems” were generated as early as 1988 and they demonstrated their usefulness in studies of hematopoiesis, basic immunology, infectious disease, and autoimmunity (Shultz et al., 2012). Creating human “organs” (such as the liver) in mice is proving its importance in drug metabolism and viral hepatitis studies (Yoshizato et al., 2012).

Mice have been widely used for genetic studies not only because their genomes are very similar to that of humans and can easily be manipulated, but also for other important reasons that make mice very close to the ideal animal model. First of all, the small size that makes the mice easy to breed in little spaces. The size also makes the mice ease to handle in daily routine procedures. A second important reason is the fact that mice reach sexual maturity very fast (between five and eight weeks of age), have high reproductive rates and relatively short lifespan. This makes it possible to easily expand experimental subjects into statistically relevant cohorts, observe different mice generations during a research, and follow the lifespan and quality of life during the whole life of the animal in a reasonable period of time. Furthermore, mice are easily available at relatively low costs and also inexpensive to raise and to maintain (Manis, 2007; Simmons, 2008). Last, but not least, what makes the mouse an attractive model is the large amount of available baseline phenotypic data (Kõks et al., 2016). Nevertheless, we always have to keep in mind that mice are not humans and despite all the progresses, strives and commitments in order to make mice always more sophisticated preclinical models, we need to know the potential limitations of extrapolating data from mice to humans.

The most obvious and evident difference is the size. Humans are roughly 2500 times larger than mice and this has an influence, for example, on the metabolic rate (Perlman, 2016). Also, gross and histologic anatomy and pathobiology differences need to be considered each time researchers investigate on specific systems (Treuting & Dintzis, 2014). Important divergences can be found in the microbiomes and pathogen susceptibility (Perlman, 2016). The literature is rich of examples of therapies that work well in mice but are not efficacious in humans (Oehler & Bicknell, 2000; Monaco, 2003; Shepherd & Sridhar, 2003; Wood, 2003). Some of these results, can be explained for example by the important differences in the immune system of mice and humans (Mestas & Hughes, 2004; Zschaler et al., 2014). Finally, if on one

side domestication and breeding are essential for biomedical research, on the other hand they have increased the differences between the biology of these strains and that of wild mice, let alone human biology (Perlman, 2016). Furthermore, the genetic homogeneity that makes these strains valuable in the laboratory means, of course, makes them lack the genetic variation that characterizes outbred wild populations (Perlman, 2016).

While it is hard to draw global significant differences between the two species, it is worth considering the possibility that any given response in a mouse may not occur in precisely the same way in humans.

2.5 C57BL/6 in Biomedical Research

When overviewing recent publications on mice, it is clear that the most widely used mice strains in BR are C57BL/6 (Bryant et al., 2008) and BALB/c. Other strains, such as A/J mice, CD1 mice, and ICR mice, are also often used. The majority of these animals are supplied by four major providers, The Jackson Laboratory, Charles River Laboratories, Taconic Farms and Harlan Laboratories.

If a research is to be reliable and reproducible over time and place, and, more importantly, if it is to have the most potential for improving human health, it must be conducted with models whose genetic backgrounds are well-defined, stable and clearly communicated. The cofounding effect of the genetic background needs to be minimized in biomedical research and some of the following tips can be taking in consideration for this purpose. First of all, mutants with genetically well-defined backgrounds should be used (Linder, 2006; Yoshiki & Moriwaki 2006). Also, the appropriate use of control animals is essential. If a mutation arose spontaneously or was induced on a well-characterized inbred strain, the inbred strain is likely coisogenic (differs at only one locus) with the mutant and therefore the best control (Linder, 2006). When possible, it is important to construct congenic, targeted mutation, transgenic and other genetically altered strains to controls that are coisogenic (Silver, 1995; Linder, 2006). It is essential to construct congenics and transgenics on well-defined backgrounds and construct targeted mutation strains on well-defined ES cell lines (Linder, 2006). Early studies using ES cells to generate gene knock-out strains of mice were limited to the “129” genetic background strain (Draper & Nagy, 2007). The phenotypes resulting from these genetic manipulations vary in function of the background strains used. Also, it is expensive and time-consuming backcrossing of founder animals in order to generate mice whose altered locus is consogenic with background strain commonly used such as C57BL/6 (Doyle et al., 2012). Fortunately, now it is possible to use ES cells derived directly from C57BL/6J or BALB/c having immediately their genetic backgrounds (Seong et al., 2004). It is important to consider the effects of environmental factors such as noise, light, home cage environment, handling and diet on gene expression and behavior (Crawley et al., 1997; Bailey et al., 2006).

In light of the above, it is easy to understand why the most used strains are only a few and that the more these strains are used, the more we will know them and their backgrounds and the more they will continue to be used. In particular, C57BL/6 mice, also called "C57 black 6" or simply "Black 6", is an inbred strain that has the advantage of being genetically stable and

that is easy to breed. As previously said, Black 6 was also the first mouse strain whose genome was fully sequenced (Mouse Genome Sequencing Consortium, 2002). The application of C57BL/6 mice consists of three main areas. The most common one is to serve as physiological or pathological models for *in vivo* experiments. Secondly, they are often applied to build transgenic mice models. Lastly, C57BL/6 mice are used as a background strain for the generation of congenics with both spontaneous and induced mutations (Johnson, 2012). Different sub-strains of Black 6 exist, and their phenotypic differences have been studied together with some of the underlying genetic alterations (Bryant et al., 2008; Mekada et al., 2009; Kumar et al., 2013).

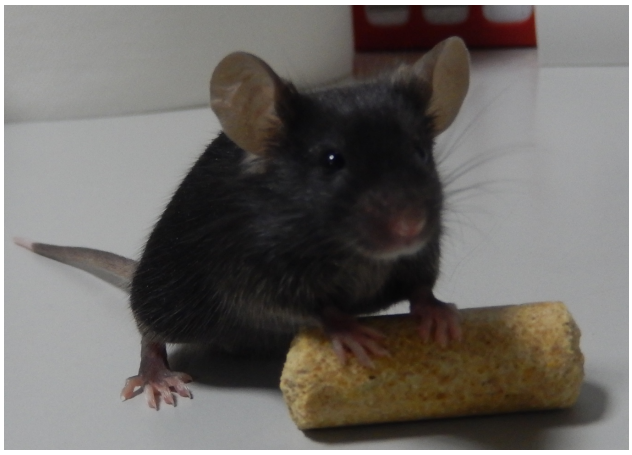


Fig. 2.5*: C57BL/6.*

C57BL/6 strain was isolated from C57BL/10 prior to 1937 and was used to establish the C57BL/6J colony at The Jackson Laboratory in 1948. Such colony was then used to originate the C57BL/6N colony at the National Institutes of Health (NIH) in 1951. In 1974, Charles River Laboratory started their own sub-colony, C57BL/6NCrl from the 32nd generation of the C57BL/6N colony (Mulligan et al., 2008). In summary, multiple branches of the C57BL/6 lineage arose across time and have been maintained as separate breeding colonies (Bryant et al., 2008). Isolation and genetic drift of these colonies resulted in the emergence of genetically distinct sub-strains. In fact, genetic drift can occur within 20 generations after separation (Bailey, 1977; Bailey, 1982), and also by random fixation of new mutations in sub-strains (Sluyter et al., 1999; Specht & Shoepfer, 2001). Many studies revealed genetic variations between both B6 lines and within the same inbred mouse strain (Bothe et al., 2004; Huges et al., 2007; Watkins-Chow & Pavan, 2008). However, the major genetic and phenotypic differences are across sub-strains more that within them. Though the phenotypes resulting

from specific allelic variants have often been characterized in detail, they can often be overlooked (Kõks et al., 2016).

C57BL/6J sub-strain from Jackson Laboratories is probably the most used inbred strain and it carries several known allelic variants that could influence phenotypic outcomes of manipulations or treatments. This strain, indeed, is described to have low susceptibility to spontaneous tumours, high susceptibility to diet-induced obesity, moderate hyperglycemia and hyperinsulinemia because of the deletion in nicotinamide nucleotide transhydrogenase that impairs glucose tolerance (Toye et al., 2005; Freeman et al., 2006). Other strain specific differences are the high susceptibility to diet-induced atherosclerosis (Paigen et al., 1987), high incidence of hydrocephalus and malocclusion, high incidence of microphthalmia and other eye defects, low bone density (Beamer et al., 1996), and late-onset of hearing loss due to an allelic variant of the *Cdh23* mutation (Johnson et al., 2006; University of Kentucky, 2017). Being Black 6 one of the most used mice in BR, it is easy to gather information on such strain. Many peer-reviewed published articles can be found and can help in the description of a new genetically modified B6 by eliminating characteristics of the background strain, therefore achieving more reliable data.

Finally, understanding the health and well-being of the mice used in research enables the investigator to optimize research results and animal care (Burkholder et al., 2012). Knowing the genetic background enables to better interpret the results of genetic engineering. Black 6 mice represent one of the best strains in our toolbox, and much translational knowledge can be gathered from the study of engineered models.

As already said, an approach using different model organisms is the key answer to further understand human disorders and to limit confusing results deriving from the model background.

2.6 Animal Welfare, Ethics and the Role of Veterinarians in Biomedical Research

Good science and good animal care go hand in hand. A sick or distressed animal does not produce the reliable results that a healthy and unstressed animal produces (Burkholder et al., 2012). For this and many other ethical reasons, "animal welfare" intended as life quality is essential. The concept of "animal welfare" encompasses not only that the animals should be healthy, well fed, and housed in an environment that they might themselves choose, but also that they should be relatively free from negative states, such as pain, fear and distress, and capable of enjoying life. Animals should also be able to carry out behaviors and activities that they are strongly motivated to do (Fraser, 2008; Fenwick et al., 2009).

Veterinarians are obviously vital in animal care. Indeed, they are significant contributors in biomedical research through the utilization of their specialized training in animal biology and medicine to model human physiology and disease. They can participate in biomedical research by directly initiating and leading research programs (principal investigators) or can contribute as co-investigators, research scientists, and technical advisors. Attending veterinarians not only provide evaluations of the care, treatment, housing, and use of all animals, but they also are a legally-mandated member of animal care and use committees. Using their specialized knowledge base, they provide technical instruction to researchers, collaborate and provide technical advice on experiments utilizing animals, and ensure animal well-being (NRC, 2004). Furthermore, they can play an important role in carrying out vigilance and monitoring of potential animal welfare impacts, especially in the research setting when new genetically engineered animal strains are being developed (Ormandy et al., 2011).

All researches implicated in animal experimentation subscribe to "The 3 Rs Principle" which stands for reduction, refinement, and replacement. Reduction refers to methods that result in fewer animals being used to acquire the needed information. This, in some studies, eliminates the use of animals. Refinement concerns the manner in which the animals are treated. This includes new and more effective anesthetics and analgesics, species-appropriate housing, and enrichment activities. Replacement means using methods that do not involve whole animals. Computer models, cell and tissues cultures are examples.

By using the "The 3 Rs Principle" to guide the ethical evaluation of animal use, the scientific community has been able to achieve important improvements in animal welfare. Today the

welfare of animals used in science is often substantially better than animals used in other ways. For example, a pig used in BR is most likely housed with a very good quality bedding and housing, will receive anaesthesia and analgesics before surgical procedures, and will have individualized veterinary care. This is in contrast with many of the living situation of typical grower pigs (Fenwick et al., 2009).

The success of the "The 3 Rs Principle" is demonstrated by its acceptance and recognition by the scientific community, humane organizations, policy makers, and the general public (Fenwick et al., 2009). Since these principles were first described by the British investigators Russel and Burch in 1959 in response to the moral and ethical concerns associated with the use of animals in research (Russel & Burch, 1959; Flecknell, 2002), they have been embedded in national and international legislation and regulations on the use of animals in scientific procedures, as well as in the policies of organisations that fund or conduct animal research. The European Directive 2010/63/EU (revising Directive 86/609/EEC on the protection of animals used for scientific purposes) and the adopted Italian legislative Decree n. 26/2014, are firmly based on "The 3 Rs Principle". With the new legislation, the scope is wider than before and includes foetuses of mammalian species in their last trimester of development and cephalopods, as well as animals used for the purposes of basic research, higher education and training (European Commission, 2017). A 'procedure' is described as "any use, invasive or non-invasive, of an animal for experimental or other scientific purposes, with known or unknown outcome, or educational purposes, which may cause the animal a level of pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice" (European Directive 2010/63/EU). The Directive lays down minimum standards for housing and care, regulates the use of animals through a systematic project evaluation requiring inter alia assessment of pain, suffering distress and lasting harm caused to the animals. It requires regular risk-based inspections and improves transparency through measures such as publication of non-technical project summaries and retrospective assessment. The Veterinarian is often directly addressed in the Directive and has a clear and important role in animal experimentation, being responsible for their welfare.

In this Directive, the development, validation and implementation of alternative methods is promoted through measures such as establishment of a Union reference laboratory for the validation of alternative methods supported by laboratories within Member States (European

Commission, 2017). In Europe, EURL-ECVAM placed at the Joint Research Centre, Institute for Health and Consumer Protection (IHCP) in Italy, at Ispra, is designated to validate, develop and spread alternative methods and to publish recommendations in order to help the adoption of these alternative methods at an international level. The Italian Ministery of Health has nominated as "single point of contact" (Art. 47, European Directive 2010/63/EU) the "Centro di Referenza Nazionale per i Metodi Alternativi, Benessere e Cura degli Animali da Laboratorio" located at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER).

The adoption of the stringent European Directive with the Italian legislative decree n. 26/2014 had a quite clear effect on animal experimentation. The Italian Ministry of Health published in April 2017 (Gazzetta Ufficiale, Serie Generale, n. 95, 24 aprile 2017) data on the use of animals for experimental purposes during the year 2015, one year after the adoption of the new law. A decrease of 15,8% of animals used for experimental purposes in comparison to 2014 was registered. Although the use of mice decreased in comparison to 2014, they still resulted the most used animals (64%) in experimental procedures, and of these 31% were used in applied and translational research. This demonstrates in some way that animals, and especially mice, will continue to serve as valuable models because unfortunately still the experimental models that are not *in vivo* are not sufficiently complex and reliable for biomedical research to go from *in vitro* and *in silico* models directly to human clinical trials. Though, alternative methods are becoming always more efficient and often adopted in order to reduce and replace animal use. However, "The 3 Rs Principle" premises that animal use for experimental research is acceptable. Indeed, there are questions that go beyond this tenant, animal welfare and health, and prompt the discussion of concepts such as *intrinsic value* of animals, which is separate from their value to humans, *integrity* or *dignity*, and *naturalness*, especially for what concerns engineered animals (Oritz & Elisabeth, 2004). In particular for genetic engineering it is hard to establish limits. On one hand, genetic engineering seems as the logical continuation of selective breeding, a practice that humans have been carrying out for years, and for most of the people human life is deemed more important than animal life. On the other hand, for some people genetic engineering can be seen as exaggerating the imbalance of power between humans and animals, and will upset the natural balance of the ecosystem (Ormandy et al., 2011). In addition, there may be those who feel strongly opposed to certain applications of genetic engineering, but more accepting of others. For example,

recent evidence suggests that people may be more accepting of biomedical applications than those relating to food production (Shuppli & Weary, 2010). Consequently, limits to genetic engineering need to be established using the full breadth of public and expert opinion. This highlights the importance for veterinarians, as animal health experts, to be involved in the discussion (Ormandy et al., 2011).

2.7 Clinical Trials

If a drug appears promising in preclinical studies, a drug sponsor can submit an investigational new drug (IND) application (in the United States) or a Clinical Trial Authorisations (CTA) application (in Europe) (Kashyap et al., 2013), that needs to be approved also by an ethics committee before phase trials are started. This application is a detailed proposal containing all kind of pre-clinical drug information and data, together with the investigator's qualifications. After approval, the drug is studied through different phase trials (described below), and if it demonstrated to be safe and efficacious, the drug sponsor can then submit a New Drug Application (NDA) to the US-FDA, or the Marketing Authorization Application (MAA) to EMA. Following an extensive review by the authorities, often involving recommendations by external committees, it is decided if the drug can be granted an indication, and marketed. Once the drug achieved the final approval, it continues to be studied in phase IV trials, in which safety and effectiveness for the indicated population is monitored. To facilitate evaluation and endorsement of foreign drug data, efforts have been made to harmonize this approval process across the United States, Europe, and Japan through the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (Umscheid et al., 2011).

In general, clinical trials encompass a number of studies that can be classified based on the objectives (human pharmacology, therapeutic exploratory, therapeutic confirmatory, therapeutic use), or on the temporal phase in which they are collocated being these phases subsequent one to the other, and named Phase I, II, III, and IV. Regardless the taxonomy employed, these phases all together give complementary data on the value of a certain drug, and the studies vary among each other in the objectives, experimental design and type of patients involved (Apolone & Garattini, 2007).

Prior experimentation and after approval of the experimental protocol by the authorities and ethics committee, people undergoing clinical trials sign an informed consent. The doctrine of the informed consent represents one of the most important changes introduced in biomedical ethics in the second half of the 20th century, and is based on people's right of self-determination. After all the atrocities seen in the "death camps" during the Second World War, were people were tortured with unapproved medical experimentation, the Nuremberg trials enshrined the principles on which human experimentation should settle. Another achievement was gained when in 1964 the World Medical Association approved with the

Declaration of Helsinki the "Recommendations guiding medical doctors in biomedical research involving human subjects". The requirements listed in the Declaration of Helsinki are now adopted and incorporated in national laws regarding human medical research (Hick, 2007).

Phase I. Phase I trials (synonymous with "dose-escalation" or "human pharmacology" studies) are the first instance in which the new investigational agent, that has been studied only *in vitro* and *in vivo* in animal preclinical studies, is studied in humans. These studies are usually performed open label and in a small number of healthy volunteers (a few dozen patients). These volunteers in a monitored environment and under strict medical control, receive single or repeated doses of the drug. Diseased volunteers are involved in phase I trials only in case of specific diseases such as malignant neoplasia where drugs are intrinsically too toxic to test on healthy volunteers and where no other therapeutic approved approaches are available. The aim of these studies is to test the safety and maximum tolerated dose (MTD) of a drug, human pharmacokinetics and pharmacodynamics, and drug–drug interactions (Umscheid et al., 2011). At the end of a well conducted phase I clinical trial different dosages have been identified, hypothesis have been made on possible negative effects of the drug, and PK/PD data is collected.

Phase II. Once phase I trials are ended, the data achieved is used to set phase II trials, also referred to as "therapeutic exploratory" trials. These trials are usually larger than phase I studies, and are conducted in a small number of volunteers who have the disease of interest. They are designed to test safety, PK, and PD in bigger population samples (few hundreds of patients), but may also be designed to answer questions essential to the planning of phase III trials, including determination of optimal doses, dose frequencies, administration routes, and endpoints. In addition, they may offer preliminary evidence of drug efficacy by comparing the study drug with "historical controls" from published case series or trials that established the efficacy of standard therapies, by examining different dosing arms within the trial, or by randomizing subjects to different arms (such as a control arm) (Umscheid et al., 2011). At the end of phase II trials safety data are confirmed, the optimal dosage to bring in phase III is identified, together with the best patients to involve in the next trial in terms of age, type and severity of the disease (Apolone & Garattini, 2007).

Phase III. This stage of drug assessment is also referred to as a "therapeutic confirmatory," "comparative efficacy," or "pivotal trial" and it is crucial for confirming the therapeutic value of the medicine candidate. It is conducted in a larger and often more diverse target population and, apart from confirming the efficacy, it should identify and estimate the incidence of common adverse reactions. However, given that these trials are usually done on 300 to 3000 subjects, they consequently have the statistical power to establish an adverse event rate of no less than 1 in 100 persons (Umscheid et al., 2011). This stage of experimentation can last 3-5 years because many aspects need to be taken into consideration and monitored, such as lifespan, life quality, symptoms etc. These trials usually compare the effect of the tested drug to a standard therapy or a placebo, other times to non-treated patients. When this stage is concluded, all data from preclinical and clinical phase I-III trials are gathered and analysed in a dossier that undergoes authority evaluation (FDA, EMA, AIFA) in order to be placed on the market (Apolone & Garattini, 2007).

Phase IV. Phase IV trials are also referred to as "therapeutic use" or "post-marketing" studies. These studies are done once a drug is approved and placed on the market. Primarily, they are observational studies aimed to recognize less common adverse reactions, and to evaluate cost and/or drug effectiveness in populations, diseases, or doses similar to or markedly different from the original study population (Umscheid et al., 2011).

In the last decade, FDA and EMA, introduced guidelines for testing 'micro-doses' (less than one-hundred of the therapeutic dose) of drugs in humans, bypassing animal models. They are known as early **"Phase 0"** studies and are used to collect human data quickly by showing how the drug is distributed and metabolized in the body, and whether it hits the right molecular target (Marchetti & Schellens, 2007).

Overall, it appears clear that the human being represents the final experimental model for the development of new therapeutic drugs. It's also evident that humans as experimental models need to be used in conditions of maximum safety, and after a thorough evaluation of the risk/benefit ratio for the people involved. It is necessary that the developmental process of a new drug undergoes accurate *in vivo* preclinical experimentation using animal models. The

current trend of proposing clinical studies of new molecules on the base of simple *in vitro* studies is not always in the interest of the diseased patients.

Biology and medicine are experimental sciences necessarily based on experimental models. As previously described these models need to be appropriate. The results, need to be significant and reproducible. In order to do so, modern research should be multidisciplinary, integrative and complementary, and should exploit advantages of each model used (Garattini, 2007).

Chapter 3

Hutchinson-Gilford Progeria Syndrome (HGPS)

3.1 Hutchinson-Gilford Progeria Syndrome (HGPS) and Aging

Aging is the natural effect of time and environment on the living organisms, and death is its end result. The physiological process of aging aims at the homeostasis of tissues through removal of cells bearing damaged genome sections or non-functional organelles (Evangelisti et al., 2016). Despite this process allows to avoid the neoplastic transformation of tissues and organs, it also causes the loss of stem cells and a reduction in extracellular components, which are also associated with inflammatory processes contributing to tissue degeneration.

The number of older persons (aged 60 years or over) has increased considerably in recent years in most countries and regions, and this trend is projected to grow in the coming decades. Between 2015 and 2030, people aged over 60 years are projected to grow by 56%, from 901 million to 1.4 billion, and by 2050, is projected to reach nearly 2.1 billion (United Nations, 2015).

Aging is the primary risk factor for numerous chronic, debilitating diseases, which impact quality of life of the elderly and their families, and consume a large portion of health care costs (Gurkar & Niedernhofer, 2015). If aging is a causal factor for such diseases is a critical question (Stewart, 2014). The growing trend of older people can be expected to have far reaching economic, social and political implications and, in particular, it puts pressure on health systems, increasing the demand for care, services and technologies to prevent and treat conditions associated with old age (United Nations, 2015). It has to be noted, that also pets', their owners and veterinarians, face a whole new set of age-related conditions since pet's lifespan has improved due to better veterinary care and dietary habits.

For all these reasons, the improvement of knowledge about the mechanisms of aging represents an important goal of current research (Michaud et al., 2013). Clinical trials to treat aging are impractical and also preclinical models to test interventions to extend health- and lifespan are lengthy and expensive (Gurkar & Niedernhofer, 2015). One useful approach is to take advantage of mouse models that are engineered to age rapidly.

Diseases exist in which individuals age more rapidly than normal. Progeric laminopathies are human syndromic diseases associated with defects of the nuclear lamina protein lamin A/C, and they represent a paradigm of age-related diseases encompassing most of the disorders linked to normal aging. This is particularly true for Hutchinson-Gilford Progeria Syndrome (HGPS), which has arisen much interest in the research community for its particular characteristics. Interestingly, HGPS and normal aging share many cellular phenotypes, such as abnormal nuclear shape, loss of epigenetic marks and increased DNA damage, as well as tissue pathologies including reduced bone density and cardiovascular disease (Burtner & Kennedy, 2010). In fact, HGPS patients exhibit accelerated atherosclerosis and die predominantly of myocardial infarction or stroke during their teenage years (Villa-Bellosta et al., 2013). Furthermore, it has recently been reported (Scaffidi and Misteli, 2006) that progerin, the mutant protein implicated in progeria pathogenesis, accumulates in tissues from physiologically aged individuals.

Therefore, although Hutchinson–Gilford progeria syndrome is very rare, improving our knowledge of HGPS and searching for innovative and efficient therapies, is extremely important because not only it could help expanding the affected childrens' lifespan and preserve their quality of life, but it may improve our understanding of aging-related disorders, in humans and animals, and the links with major physiological processes such as those involved in oncogenesis (Cau et al., 2014).

3.2 History of HGPS

In 1886, Jonathan Hutchinson described for the first time a 3-years old boy with what for him was an ectodermal dysplasia (Hutchinson, 1886), and in 1895 he wrote a second brief report on a second patient. A few years later, Hastings Gilford described in detail these two patients and suggested to use the word "progeria" for this disease (Gilford, 1897; Gilford, 1904). The word "progeria" comes from the ancient Greek, *pro* meaning "before" and *geras* meaning "old age".

Hutchinson-Gilford Progeria Syndrome (HGPS), as its name suggests, involves premature aging in children. In this syndrome, the rate of aging is accelerated up to seven times that of normal (Rastogi & Mohan, 2008). However, patients with HGPS do not show all features usually associated with aging, such as increase in cataract formation or cognitive degeneration, therefore HGPS has been referred to as a ''segmental'' progeroid syndrome (Martin & Oshima, 2000). HGPS normally leads to death at an average age of about 13 years, usually due to stroke or myocardial infarction (Merideth et al., 2008; PRF, 2017a); however, in some cases children died as early as 7 and some have survived till the age of 30 (Shankar et al., 2010).

The interest in HGPS increased in 2003, when mutations in the gene encoding lamin A/C (*Lmna*) was discovered to be the cause of the disorder (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Before 2002, mutations at this level were already known to cause at least five different entities: Autosomal Dominant Emery-Dreyfuss muscular dystrophy, limb-girdle muscular dystrophy type IB, dilated cardiomyopathy type 1A, Autosomal Recessive Charcot-Marie-Tooth type 2B1, and Familial Partial Lipodystrophy Dunnigan type (Hennekam, 2006).

3.3 Epidemiology of HGPS

HGPS is a rare, fatal genetic condition characterized by features reminiscent of marked premature aging in children. The reported prevalence rate of the disease is one in eight million births, but if unreported or misdiagnosed cases are taken into account, the estimated birth prevalence is one in four million (Coppedè, 2013). Presently, there are 145 identified patients living with progeria in 46 countries (**Fig. 3.1**), 33 of which have a mutation in the lamin pathway but do not produce progerin (PRF, 2017a). The ratio of females to males is 1:1.5 and 97% of patients are white, however there is no explanation for this tribal discrepancy (DeBusk, 1972). This disease is generally not heritable because patients hardly reach the reproduction stage. Genetic studies report that the most possible form of inheritance is a periodic autosomal dominant mutation on the fertilizing ovum or sperm (Brown et al., 1985; Brown, 1987; Beauregard & Gilchrest, 1987). Normally, healthy patients do not pass it on to their children because it is genetically dominant (Korf, 2008; Shankar et al., 2010). To date, there are only two cases in which it became evident that a healthy parent can carry the *Lmna* mutation that causes progeria: one is a family from India that have five children with Progeria, the other one is a family from Belgium with two (Shankar et al., 2010).

Fig. 3.1: *Map indicating HGPS distribution around the world. This figure includes 112 children with classic Hutchinson-Gilford Progeria, all of whom have a progerin producing mutation in the Lmna gene, and 33 children in the progeroid laminopathy category who have a mutation in the lamin pathway but do not produce progerin (Adapted with permission and courtesy of the Progeria Research foundation https://www.progeriaresearch.org/meet-the-kids/).*

3.4 Molecular Background, Cause and Pathogenesis of Progeria

Many researchers screened progeria-affected patients and discovered that the disease is associated with mutations in the *Lmna* gene. The official name of this gene is "lamin A/C" and is also known as LMN1, LMNA_HUMAN and LMNC (Shankar et al., 2010). *Lmna* gene is located on the long (q) arm of chromosome 1, between position 21.2 and 21.3 (cytogenetic location: 1q21.2-q21.3). The molecular position of *Lmna* is on chromosome 1:156, 084,460 to 156,109,877 base pairs (Shankar et al., 2010). The nuclear lamins are type V intermediate filament proteins that are critically important for the structural properties of the nucleus (Dechat et al., 2010). Mutations in lamin proteins, primarily in the *Lmna* gene, are associated with over a dozen degenerative disorders (Gonzalo et al., 2017). These diseases are known as laminopathies and encompass a range of phenotypes with different tissue-specific pathologies, including muscular dystrophy disorders (e.g., Emery-Dreyfus Muscular Dystrophy or EDMD, Limb-Girdle Muscular Dystrophy Associated with Atrioventricular Conduction Disturbances or LGMD1B), peripheral neuropathies (e.g. Charcot-Marie-Tooth-Disease type 2B1 or CMT2B), lipodystrophies (e.g. Familial Partial Lipodystrophy of the Dunnigan Type or FPLD), as well as systemic laminopathies such as Hutchinson Gilford Progeria Syndrome (HGPS), Atypical Werner Syndrome (AWS), Mandibuloacral dysplasia (MAD) and restrictive dermopathy (RD) (Worman et al., 2009; Vigouroux & Bonne, 2013; Gordon et al., 2014; Gonzalo & Kreienkamp, 2015; Vidak & Foisner, 2016; NIL, 2017).

The *Lmna* gene encodes four type A lamins (A, C, CΔ10, and C2) via alternative splicing. Lamin A and C, identical up to residue 574, are the most ubiquitously expressed. Lamin C possesses five unique C-terminal residues, and lamin A is synthesized as a 664-residue prelamin A precursor that after post-translational processing results in a mature lamin A protein of 646 residues. Prelamin A undergoes a process of maturation involving the CAAX box at the carboxy terminus. At the first stage of maturation farnesyl transferase adds a farnesyl group to the cysteine. Next, either Rce1 (FACE2 in human beings) or *Zmpste24* (FACE1 in human beings) cleave the last three amino acids (AAX). At the next stage the isoprenylcysteine O-methyltransferase (ICMT) methylates the farnesylated C-terminal cysteine. Another cleavage event of the last 15 C-terminal amino-acids by *Zmpste24* is necessary to remove the carboxy farnesylated and methylated cysteine (Corrigan et al., 2005; Coppedè, 2013; Gonzalo et al., 2017).

Lamin A is a scaffold protein located between the nuclear membrane and the peripheral chromatin and represent one of the building blocks of the nuclear envelope (Prokocimer et al., 2009). Lamins and their associated proteins are involved in maintaining the shape and mechanical strength of the nucleus. They are also involved in most nuclear activities, including chromatin organization, DNA replication, transcription regulation, RNA processing, linking the nucleus to all major cytoskeleton networks, apoptosis, meiosis and mitosis (Prokocimer et al., 2009; Shankar et al., 2010), although the molecular details of some of these functions need to be better elucidated (Rusiñol & Sinensky, 2006). Overall, it can be stated that lamin A broadly influences nuclear structure and function (Broers et al, 2006). Schematic representations of the structure of lamin proteins, nuclear envelope, lamina and chromatin and of prelamin A are shown in **Fig. 3.2, 3.3** and **3.4**, respectively**.**

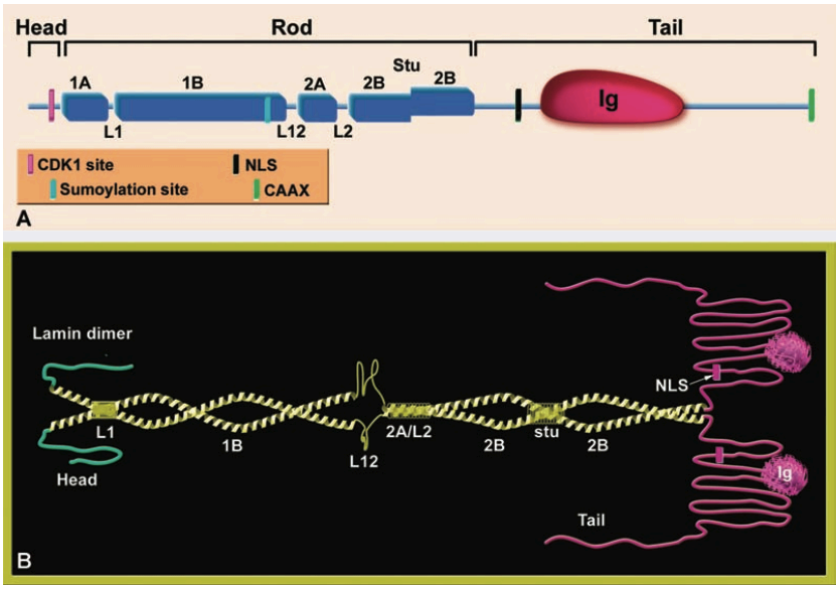


Fig. 3.2: *Schematic representation of the structure of lamin proteins.* ***(A)*** *The lamin monomer is divided into three domains, head, rod and a globular tail. The rod domain is composed of four coiled-coil regions (1A, 1B, 2A, 2B) that are connected through three short linkers (L1, L12, L2). Marked on the scheme are the Ig globular domain in the tail and the stutter (a discontinuity of the heptad repeat) in coil 2B. Also shown by colour code are the positions of the CDK-1 recognition site (absent in Ce-lamin), the sumoylation site in human lamin A, the nuclear localization signal (NLS) and the CAAX motif.* ***(B)*** *A model of lamin dimers. A pair of parallel coiled-coil rods forms the lamin dimer (yellow). The non-helical head and tail domains are coloured green and pink, respectively. The different sub-domains are indicated. In coil 2B the stutter leads to a local unwinding (Prokocimer et al., 2009; reproduction licensed).*

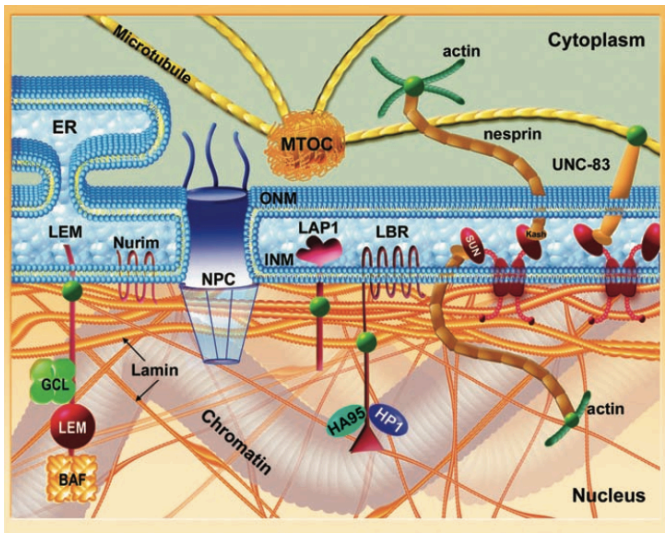


Fig. 3.3: *Schematic view of the nuclear envelope, lamina and chromatin. The inner nuclear membrane (INM) and the outer nuclear membranes (ONM) are joined at the nuclear pore complexes and separated by the nuclear lumen. The ONM and lumen are continuous with the endoplasmic reticulum (ER). Lamins (both A- and B-types) are shown as orange filaments; thicker at the nuclear periphery and thinner filaments in the nucleoplasm. However, the filamentous nature of the lamins, especially within the nucleus, remains hypothetical. Also shown are selected proteins of the INM including LEM-domain and SUN domain proteins, LAP-1, Nurim and LBR (boudreaux). These proteins represent only a small fraction of proteins of the INM. Also shown few examples of non-integral proteins that interact with lamins or with their associated proteins including actin, HP1, HA95, germ cell-less and BAF. The nucleoplasmic lamins also form specific protein complexes (not shown). INM SUN-domain proteins interact with ONM KASH-domain proteins, thus bridging between the nucleus and cytoplasmic structures including, actin (green), tubulin (yellow) and intermediate filament (not shown) networks and the centrosome (MTOC) (Prokocimer et al., 2009; reproduction licensed).*

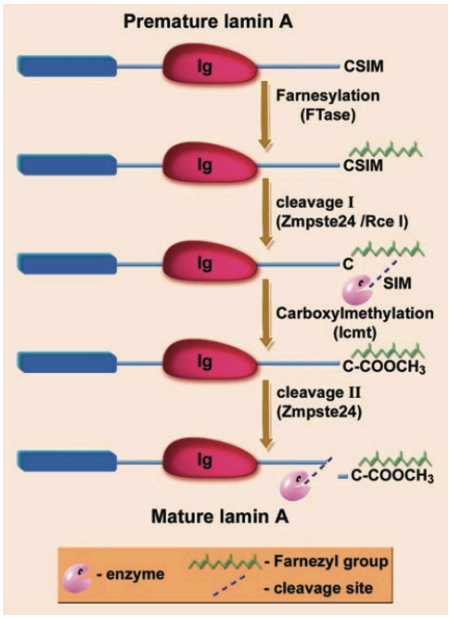


Fig. 3.4: *Prelamin A processing. Premature lamin A is going through four processing steps until it becomes a mature lamin A, including farnesylation of the cysteine at the carboxy terminus, cleavage of the three carboxy-terminal amino acids (aaX) by either Zmpste24 or RceI, carboxymethylation of the farnesylated cysteine by isoprenylcysteine methyltransferase and cleavage of the 15 terminal amino acids, including the farnesylated and carboxymethylated cysteine, by Zmpste24 (Prokocimer et al., 2009; reproduction licensed).*

In progeria patients, the most frequent detected mutation is a *de novo* autosomal dominant, single base substitution in *Lmna* gene at position 1824 (written as C1824T). Specifically, in over 90% of progeria patients at codon 608 the cytosine is substituted with a thymine in exon 11 (Shankar et al., 2010). This mutation in also noted as Gly608Gly or G608G, which is the position in the lamin A protein affected. Less frequently, other mutations were detected in HGPS patients, such as E145K, G608S, A57P, L140R, T528M, R527C, R644C, E145K, K542N, R471C, G608S, R133L, M540T, and T623S (Taimen et al., 2009; Ahmed et al., 2017). It still has to be determined how progeria arises from these other progeric mutations, as it is unclear if these mutations result in post-translational processing defects and/or retention of the farnesyl group (Stewart, 2014).

G608G mutation is silent, not causing changes at the aminoacid level, though it activates a cryptic splice site that produces a mutant prelamin A protein with an internal deletion of 50 aminoacids. This truncated prelamin A, termed progerin, is missing the second *Zmpste24* cleavage site, leaving the mutant protein permanently carboxy farnesylated and methylated, which cannot then mature to lamin A (**Fig. 3.5**).

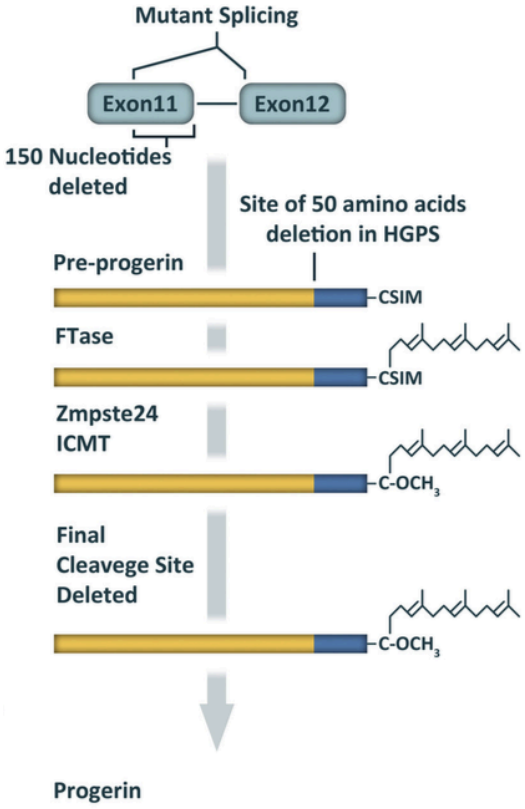


Fig. 3.5: *Mutant splicing in HGPS patients. A mutation in exon 11 activates a cryptic splice site leading to deletion of 50 amino acid residues from the precursor protein, including the final Zmpste24 cleavage site, and accumulation of farnesylated progerin (Gonzalo et al., 2017; reproduction licensed).*

Progerin is incorporated into the lamina and changes the structure of the macromolecular interaction, intra-nuclear architecture, and nuclear lamina that collectively have a prime effect on nuclear function (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003; Prokocimer et al., 2009; Ahmed et al., 2017) (**Fig. 3.6**). Primary fibroblasts from HPGS patients show drastic alterations (**Fig. 3.7**) in the nuclear shape, including loss of peripheral heterochromatin, thickened lamina, lobulation of the nuclear envelope, and clustering of nuclear pores (Hutchison et al., 2001; Scaffidi & Misteli, 2005). Existence of progerin alter mitosis that leads to changed histone modification patterns, downregulation of several nuclear proteins (Cao et al., 2007), mis-segregation of chromosome (McClintock et al., 2007), and DNA repair disorders (Manju et al., 2006). Progerin was found to cause defects in chromosome segregation in metaphase, to trap lamina components, and delay nuclear envelope reformation and the inner nuclear envelope proteins of the endoplasmic reticulum at the end of mitosis. In addition, progerin was also noted to relocate the centromere protein F from metaphase chromosome kinetochores causing binucleated cells, increase chromatin lagging and genome instability. Accordingly, accumulation of progerin-dependent defects with every round of mitosis leads cells towards premature senescence (Eisch et al., 2016). It is still not completely understood how all these changes lead to the signs and symptoms of HGPS and the reason of similarities and differences between HGPS phenotypes and those of aging. It is interesting that the cryptic splice site activated in HGPS to create progerin is also used at low frequencies in healthy individuals since increased progerin levels are also found in normal aging cells (Scaffidi & Misteli, 2006; McClintock et al., 2007; Prokocimer et al., 2013).

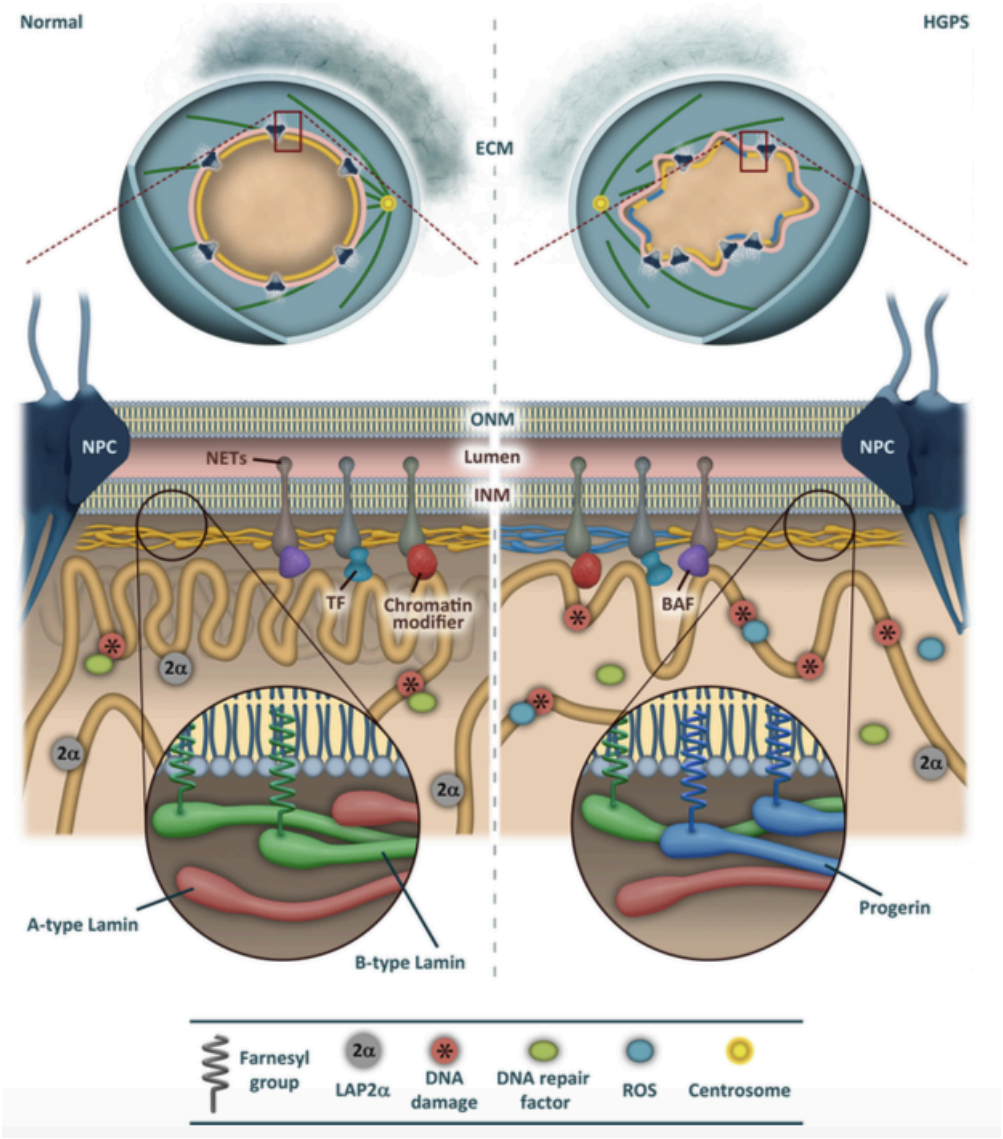


***Fig. 3.6**: Expression of progerin alters nuclear organization and genome stability. Cells from HGPS patients are characterized by a series of alterations including reduced expression of extracellular matrix (ECM) components, nuclear envelope blebs, clustering of nuclear pore complexes (NPC), loss of peripheral heterochromatin, and reorganized microtubules. Progerin expression also affects dynamics of nuclear envelope transmembrane proteins (NETs), including emerin, and their interactions with chromatin-associated proteins, such as BAF, transcription factors (TF) and chromatin modifiers. HGPS cells have higher levels of reactive oxygen species (ROS) and DNA damage, whereas LAP2 is downregulated (Gonzalo et al., 2017; reproduction licensed).*

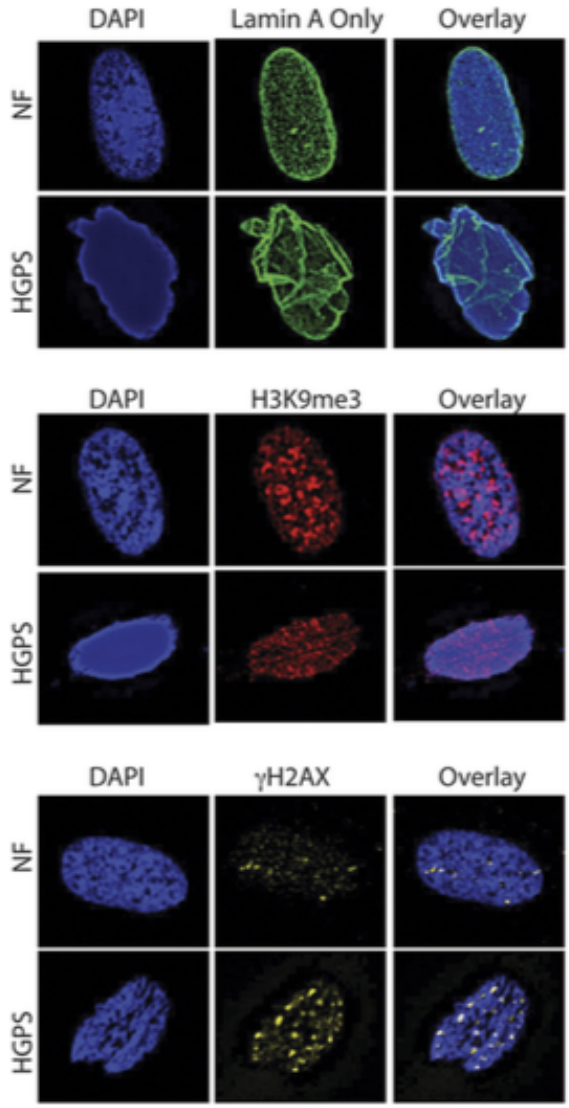


***Fig. 3.7**: Nuclear defects in HGPS cells.*
Immunofluorescence performed in primary normal human fibroblasts (NF) and HGPS patient derived fibroblasts (HGPS) with antibodies recognizing lamin A (green, top panels), histone modification H3K9me3 (red, medium panels), and H2AX (yellow, bottom panels), a marker of DNA damage. DAPI staining was used to demarcate nuclei (blue staining in all panels). Note how HGPS patient derived fibroblasts exhibit nuclear morphological abnormalities, decreased levels of H3K9me3, and accumulation of basal levels of unrepaired DNA damage, when compared to normal fibroblasts (Gonzalo et al., 2017; reproduction licensed).

3.5 Clinical Features in HGPS

HGPS is normally diagnosed between the first and second year of life, although some typical features indicate the presence of the disease at birth. In fact, progeria develops a characteristic facial appearance including prominent eyes, a thin nose with a beaked tip, thin lips, a small chin, protruding ears with lacking lobules and facial cyanosis (due to insufficient blood oxygenation). It must be said that a characteristic visible vein across the nasal bridge is often the earliest symptom (**Fig. 3.8**), followed by the scalp veins (Hennekam, 2006).

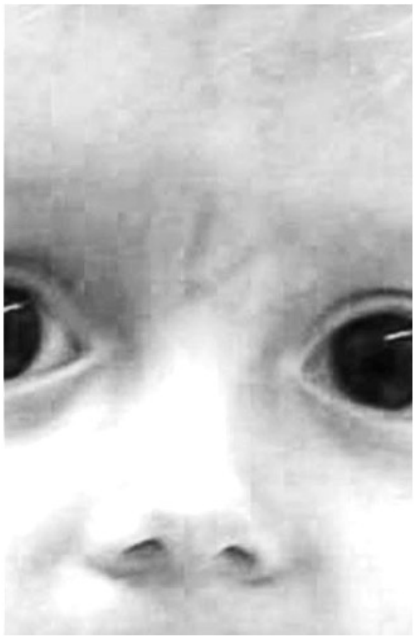


Fig. 3.8: *Prominent vein across the nasal bridge is often the first symptom in patients (Hennekam, 2006; reproduction licensed).*

Slow growth rates appear sharply already during the first year of age; in particular height deficit, and, even more, weight deficit, are particularly suffered and the most severe changes occur after 2 years of age. On average, HGPS children gain 0.4–0.5 kg/year (Coppedè, 2013), and the weight curve runs almost horizontally from the age of 2 years (Hennekam, 2006). The elderly patients have a mean weight of 14.54 kg with a mean height of 109 cm.

Intra-abdominal fat in progeria children appears with a prominent belly. The loss of subcutaneous fat, typical of the disease, starts from six months of age and becomes more evident around 3-4 years of age. Such loss is primary seen in the limbs and thorax and then in the face. The buccal and pubic fat disappear last (Hennekam, 2006). Vanishing of intra-orbital and subcutaneous fat leads to the prominent appearance of eyes (no true exophthalmos is found), skin becomes thin, and blood vessels become more visible (Hennekam, 2006).

Craniofacial abnormalities (**Fig. 3.9; Fig. 3.12**) are constantly seen in HGPS patients. In one study, thinning of the calvarium was seen in 95% of the individuals, in 91% of the cases accompanied by a paucity of scalp fat (Ullrich et al., 2012). A mottled appearance of the skull was seen in 59% of the patients. Two individuals had skull fractures, and prominent vascular markings of the bony calvaria were observed in 90% of the subjects. Craniofacial disproportion and a J-shaped sella were observed in almost 90% of the patients. Also, delayed closure of the anterior fontanel was seen in more than half of the cases (56%). Concerning oral maxillary, zygomatic arch, and parotid gland features, the authors observed a short mandibular ramus

in 83% of the patients, with a gracile thin zygomatic arch in 50% of them. A shallow glenoid fossa with a hypoplastic or absent articular eminence and flattening of the mandibular condyle were seen in 43% of the patients (Ullrich et al., 2012). In another study, 45% of the children had a V-shaped palate, and 50% of them had disorganized dentition (Schmidt et al., 2012). Oral abnormalities such as two rows of teeth, ogival palate, ankyloglossia, delayed tooth eruption, vertical chewing where rotatory chewing is supposed to develop, hypodontia were noted, as also limited size of mandible and maxilla dental crowding (Batstone & Macleod, 2002; Merideth et al., 2008). Generally, patients have a high-pitched voice. A prominent parotid gland was seen in all the children analysed. With regard to orbital features, Schmidt et al. (2012) reported hypotelorism in 86% of the children, and kinking of the optic nerves in 89%.

Fig. 3.9: *Detail of face showing thin facial skin with excessive folding on forehead and cheeks, pseudo-protrusion of the eyes, thin nasal bridge, and collapsed, flattened and broad nasal tip (Hennekam, 2006; reproduction licensed).*

Between 6 and 12 months of age kids have swollen and thick skin with oedema. With time, skin becomes sclerodermatous (**Fig. 3.10A**) and erect and finally (between 6 month and 2 years of age) the skin become dry (sometimes with fine scaling), thin, and atrophic. Sometimes, it is slightly erythematous. Many wrinkles are observed as also pigmented aged

spots (**Fig. 3.10B**). Skin over the bone fingers and toes becomes red and swollen, and the nails become dystrophic (**Fig. 3.11**) (Merideth et al., 2008).

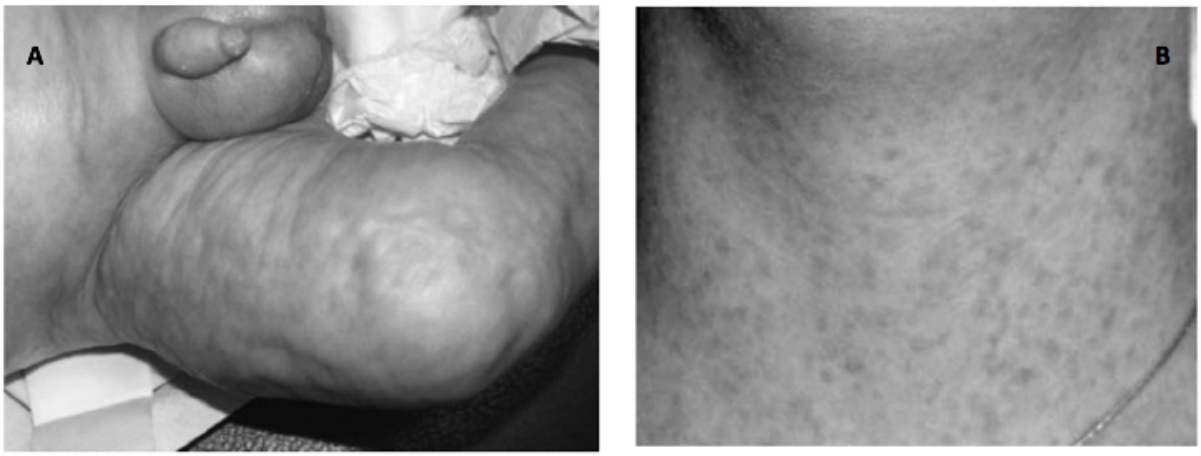


Fig. 3.10: A*) Patient at 6 weeks of age showing early phase scleroderma. Note swollen skin, pitting, and predilection localization (lower abdomen, genitalia, upper legs).* ***B)*** *Hyperpigmentation. Small, spotty pigmentations with a café-au-lait color or somewhat darker. Note predilection localization (neck, upper thorax); later on, hyperpigmentation can also be seen on the scalp (Hennekam, 2006; reproduction licensed).*

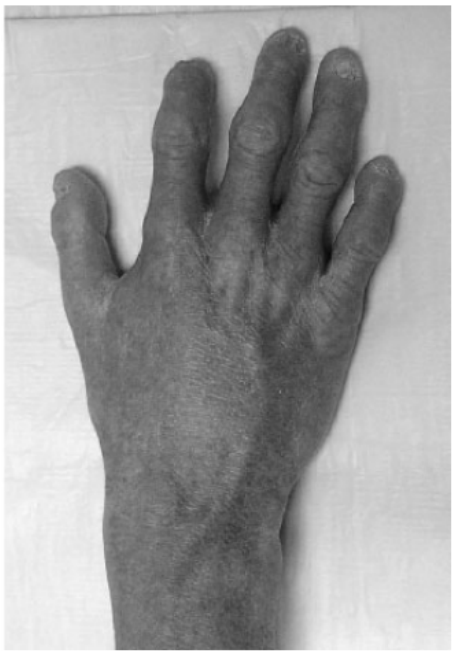


Fig. 3.11: *Camptodactyly (fingers permanently bent) in a 7-year-old patient with dystrophic nails (Hennekam, 2006; reproduction licensed).*

Another persistent characteristic of HGPS is progressive alopecia (loss of hair, eyelashes and eyebrows) with few soft hairs persistent, usually uncoloured (**Fig. 3.12**). Alopecia usually takes place within 6 months to 2 years, and between the ages of 2 years and 3 years most children become bald (Coppedè, 2013).

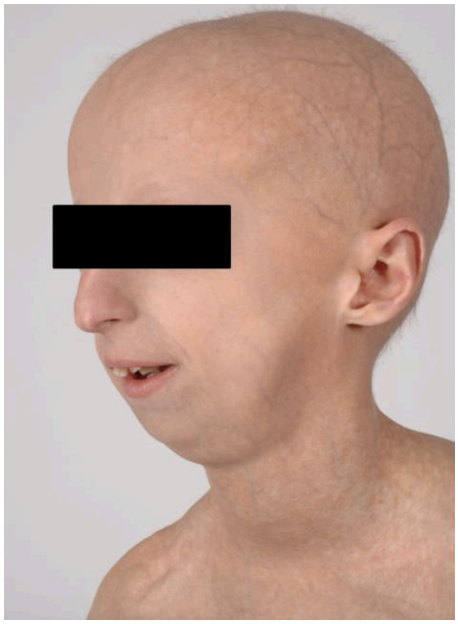


Fig. 3.12: *A typical Hutchinson-Gilford progeria syndrome "inverted triangle" face with a disproportionately hypoplastic midface and mandible. Alopecia (Guardiani et al., 2011; reproduction licensed).*

Cataracts have not been found in patients with HGPS. Strabismus and mild myopia is not uncommon. Unusual eye findings have been irregular nystagmoid movements, ptosis and Marcus–Gunn phenomenon, retinal arteriolar narrowing and tortuosity, photophobia and excessive ocular tearing (Hennekam, 2006; Coppedè, 2013; Goyal et al., 2014). Fifteen patients with HGPS have been enrolled in a prospective study to evaluate otologic and audiologic manifestations (Guardiani et al., 2011). All patients had small or absent lobules (**Fig. 3.13**), stiff auricular cartilages, hypoplasia of the lateral soft-tissue portion of the external ear canal that led to a shortened canal. A low-frequency conductive hearing loss in the 250 Hz to 500 Hz range was observed in 86.4% of the ears, despite largely normal tympanometry. In addition, 71% of the patients had dry cerumen impaction, and 29% of them reported a history of recurrent otitis media (Guardiani et al., 2011).

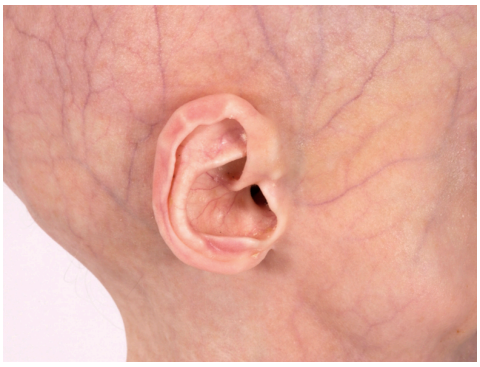


Fig. 3.13*: A typical Hutchinson-Gilford progeria syndrome pinna with stiff cartilages and a small lobule (Guardiani et al., 2011; reproduction licensed).*

Joint and bones abnormalities are common (**Fig. 3.14**), and often hip dislocation and fractures are observed in progeria patients. Joint mobility is normal at birth but decreases between 2 and 3 years, initially in the knees followed by the elbows and fingers. Children develop a widebased, shuffling gait, caused by the combination of *coxa valga* and joint stiffness. At first, the muscles appear prominent due to the disappearing of subcutaneous fat, and then the muscle bulk also decreases. Some children present a torticollis and a cervicothoracic kyphosis. Moreover, with time osteopenia of the long bones develops. The basis of the joint and muscle problems in progeria has rarely been studied (Hennekam, 2006).

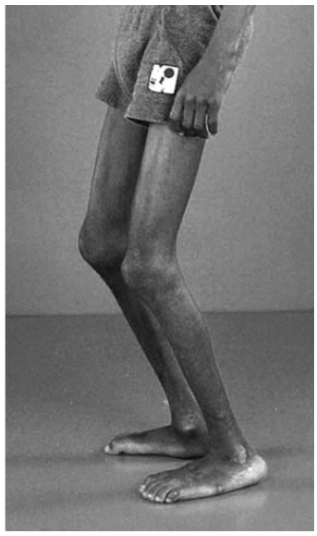


Fig. 3.14: *Legs of 8-year-old Dutch Patient 3. Note flexed knees, prominent joints, and decreased subcutaneous fat tissue. The mobility in the ankles was already severely limited (Hennekam, 2006; reproduction licensed).*

A comprehensive survey of the skeletal dysmorphisms observed in children with HGPS using conventional radiography was obtained from 39 children with the classic HGPS genotype (Schmidt et al., 2012). All patients had small clavicles, followed by *coxa valga* (**Fig. 3.15A**) and acroosteolysis, which were observed in more than 90% of the patients, and resorption of the distal clavicles and narrow apices, both present in 82% of the subjects (Schmidt et al., 2012). In general, it can be stated that osteolysis is often found at the distal phalanges (**Fig. 3.15B**), clavicles, mandible, neurocranium and viscerocranium of some patients. In classical HGPS, osteolysis seems to be restricted to these bones; however, there are also reports involving the first ribs. With the increment in mandibular osteolysis, retrognatia occurs (Ahmed et al., 2017). During the first two years of life, the size of the chin shrinks, as also shoulders with a gradual narrowing of the upper part of the body (Rastogi & Mohan, 2008; Ahmed et al., 2017).

The skull of patients appears giant-sized in contrast to the body and face (**Fig. 3.16C**), and often fractures at this level occur during the final stage of the disease (Hennekam, 2006; Ahmed et al., 2017).

No male patient is known to have fathered a child but a 23-years old woman with non-classical progeria gave birth to a healthy child (Corcoy et al., 1989). Breast development is usually completely absent. Marked hypoplasia of the nipples (but not true athelia) has been described several times. Menarche has been reported at 14 years, with subsequent irregular cycle (every 2–3 months) (Hennekam, 2006). The mail reproductive organ remains small, with testes normally descended (Ahmed et al., 2017).

Mental development and motor skills such as sitting, standing and walking are not affected. Children with progeria are often remarkably alert, active, cheerful with a normal psychosocial growth (Ahmed et al., 2017). HGPS patients do not display neurological aging to any significant extent (Mitchell et al., 2015), suggesting that the brain may be protected from, and/or insensitive to, and/or unaffected by, the expression of the progeria mutation (Baek et al., 2015).

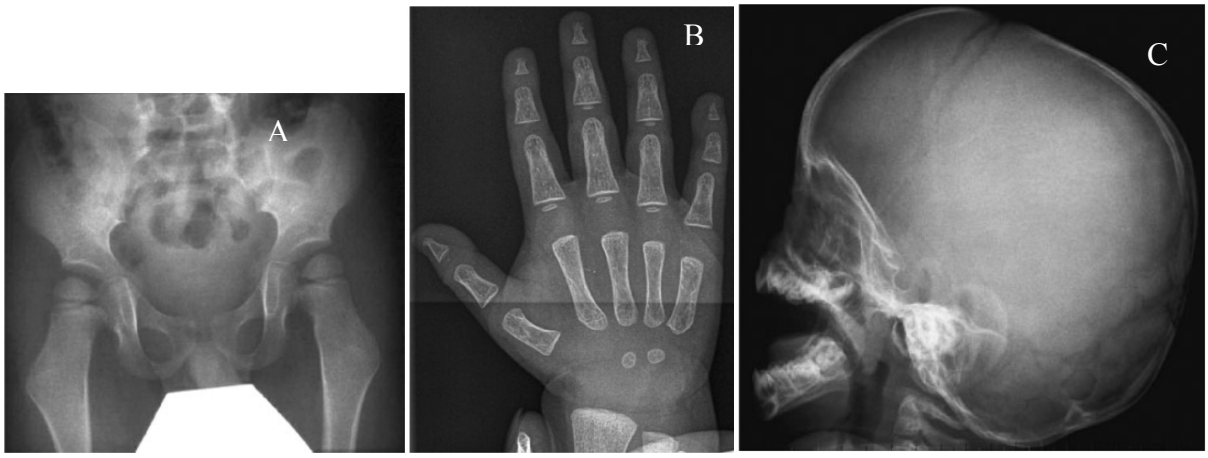


Fig. 3.15: *Human HGPS patient x-rays.* ***A)*** *Coxa Valga;* ***B)*** *Osteolysis of the distal phalanx of the 2nd and 5th finger;* ***C)*** *Relatively large neurocranium compared to the viscerocranium (especially the mandible), open anterior fontanel, thin cranial vault, and mild wormian bone formation at the occiput (Hennekam, 2006; reproduction licensed).*

A rare case of a 10-year-old boy with classical HGPS with hypoparathyroidism has been reported in Egypt (Kalil & Fargalley, 2012). Insulin resistance is common, occurring in about 50% of affected patients, however without progression to diabetes mellitus (Coppedè, 2013). Cardiovascular disease (CVD) represents the main factor affecting mortality in HGPS individuals (myocardial infarction, stroke, congestive cardiac failure). Merideth et al., (2008) observed that few children had increased systolic and diastolic blood pressure. At 6-8 years of

age, affected children slowly develop shortness of breath with easy fatigability and exertion, when blood pressure and pulse rates increase. Heart problems come into view sharply after birth and finally leading to abrupt death. The rapid progression of CVD in HGPS presents an opportunity to explore the natural history of human CVD, and a study performed on 26 HGPS patients and 22 matched controls revealed that the carotid–femoral pulse wave velocity was dramatically elevated in patients (Gerhard-Herman et al., 2012). Carotid duplex ultrasound echobrightness, assessed as a measure of arterial wall density, was significantly greater than age- and sex-matched controls in the intima–media, near adventitia, and deep adventitia, as was internal carotid artery mean flow velocity. Overall, those data demonstrated that, along with peripheral vascular occlusive disease, accelerated vascular stiffening is an early and pervasive mechanism of vascular disease in HGPS.

In fact, the most evident post-mortem characteristic are cardiovascular abnormalities. Particularly, advanced coronary atherosclerotic lesions have been reported, with arteries frequently stenosed or occluded by plaques or narrowing of intramural arteries. Occlusion of the right coronary artery, lesions of the left anterior descending artery, and severe atherosclerosis of the aorta appear to be common (Qi & Xie, 2013). Valvular changes and pulmonary arterial lesions have also been reported in HGPS individuals (Qi & Xie, 2013).

A recent cohort study that included 25 children with HGPS aimed to identify the neurovascular features, infarct type, topography, and natural history of cerebrovascular arteriopathy and stroke in these patients (Silvera et al., 2013). Neurovascular imaging revealed a unique vasculopathy, including distinctive intracranial steno-occlusive arterial lesions, basal cistern collateral vessels, and slow compensatory collateral flow over the cerebral convexities. Moreover, the authors identified early and clinically silent strokes as a prevalent disease characteristic in HGPS. Indeed, a radiographic evidence of infarction was found in 60% of patients, of which half were likely clinically silent (Silvera et al., 2013). Cerebral obstruction of blood supply may result in dysarthria, hemiplegia, and seizures (Dyck et al., 1987).

Overall, the first cause of death is stroke, observed at the mean age of 9 years. Mean age of death is approximately 13 years, with a range of 7-30 years (Debusk, 1972; Hennekam, 2006; Rastogi & Mohan, 2008; Shankar et al., 2010; Ahmed et al., 2017). However, other death reasons are reported, such as infections (Ishii, 1976), pulmonary hypertension (Shiraishi et al., 2001), complications of cardiac surgery (Corcoy et al., 1989), intracranial bleeding (DeBusk, 1972; Stehbens et al., 1999), and convulsions (Gabr, 1960).

3.6 Aging, Cancer and Lamins

It is interesting to note that comparative analysis of data on cancer incidence indicate that after a steady increase during adult life, the cancer incidence rate slows down at old age (above 70) for most sites of cancer development, as well as for all cancers combined (Anisimov et al., 2005). This phenomenon has been explained by detection bias, survival selection with old age of individuals that are less prone to cancer, and somatic aging. Ukraintseva and Yashin (2001, 2003) suggested that somatic aging might create conditions that oppose cancer development in older patients. First, the decline in the rates of metabolism, information processing and cell proliferation in an aging organism, might slow down the accumulation of some pathological changes in the human body. Second, the risk of cancer could diminish in the old simply because the proportion of senescent cells, less prone to malignant transformation, increases. Third, the physiological and metabolic changes that accompany ontogenetic transitions in an organism (for example, switching off reproductive function at the menopause) might change the spectrum of internal cancer-risk factors, resulting in decreasing vulnerability to some cancers. In is interesting to note, that also in HGPS patients there is not increased incidence in cancer. This case, is different from other premature aging syndromes, such as Werner syndrome, or others caused by mutations of DNA repair genes (Coppedè, 2013). Scientists from the National Cancer Institute (NCI) found that cells from HGPS patients contain a tumour protection mechanism that is mediated by bromodomain-containing protein 4 (BRD4), a protein that is encoded by the BRD4 gene (NCI, 2014). In literature, osteosarcoma was described in two patients and was explained on the basis of a rearranged p53 gene implicated both in HGPS syndrome (Varela et al., 2005) and osteosarcoma cells (Masuda et al., 1987; Shalev et al., 2007). Indeed, accelerated senescence appears to be partially ameliorated by deleting p53, demonstrated by the fact that *in vivo* deletion of p53 improved the postnatal growth and viability in a mouse model of progeria (Varela et al., 2005). However, excluded the above-mentioned case, no other progeria children were reported to suffer from cancer.

In general, nuclear laminas are currently emerging as an additional event involved in malignant transformation. The presumed association is multifactorial and affects tumorigenesis from its initial step to its advanced stage of metastatic spread. Lamins involvement in cancer-associated processes has been related, mainly, to their role as guardian of the nuclear architecture, their role in regulating basic nuclear activities that are implicated

in tumorigenesis, their multiple interactions with major cancer gene pathways and their role in chromosomal segregation control with its resultant impact on aneuploidy (Prokocimer et al., 2009). Studies have reported differential expression of A-type lamins in tumour tissues and have linked their absence to increased proliferation in a range of cancers, including ones of the skin, lung, thyroid, lymphatics and soft tissue (Stadelmann et al., 1990; Broers et al., 1993; Venables et al., 2001; Tilli et al., 2003; Wang et al., 2015). Until Willis et al. (2008), no study was able to link either absence or presence of A-type lamins to tumour progression, although Venables et al. (2001) suggested that loss of expression of lamins A/C was correlated with enhanced proliferation rates in tumours and that downregulation of lamin A might have been a requisite of tumour progression. Willis et al. (2008) reported that lamin A but not lamin C is a potential biomarker of the stem cell niche in the colonic crypt and secondly, expression of lamin A/C in colorectal cancer (CRC) tissues is strongly correlated with mortality. Therefore, lamin A/C may represent a novel and important prognostic biomarker in CRC. Also, Wang et al. (2015) suggested that lamins might have some value in diagnosing thyroid tumours.

It has also been reported that progerin is expressed in a number of human cancer cell lines and promotes tumorigenesis by increasing genomic instability in cancer cells (Tang et al., 2010).

On the basis of all the aforementioned, it is plausible to think that predisposition to cancer in HGPS might be in most cases masked by the short lifespan of patients (Shalev et al., 2007) and could be a potential issue if lifespan of patients is increased.

Furthermore, given the efficiency of farnesyltransferase inhibitors (FTIs) in the nuclear architecture rescue of human HGPS fibroblasts and a broad spectrum of human cancer treatments, the targeting of progerin may open a new avenue for human cancer therapy (Sepp-Lorenzino et al., 1995; End et al., 2001; Capell et al., 2005; Young et al., 2005; Tang et al., 2010).

3.7 Diagnosis of HGPS

Until 2003, there was not any definitive test to diagnose progeria. Diagnosis was based on the observation of the typical phenotype, as well as x-rays and urinary hyaluronic acid testing. It must be clear that signs are not fully evident until a child's first or second year of age, and that some signs of HGPS can be confused with other progeroid syndromes. Therefore, misdiagnosis was a frequent event (PRF, 2017b).

The measurement of hyaluronic acid in the urine, reported to be increased in HGPS children, was used for many years in diagnosis but it is now considered unreliable (Gordon et al., 2003). With the discovery of the cause of HGPS in *Lmna* gene, patient's blood samples and a skin biopsy can be evaluated for the presence of the mutated gene. This, not only gives a definitive diagnosis, but also translates into earlier diagnosis and early medical intervention (PRF, 2017b).

Also, prenatal diagnosis is possible by analysis of DNA extracted from foetal cells obtained by amniocentesis usually performed at about 15-18 weeks' gestation or chorionic villus sampling at about 10-12 weeks of gestation. Since recurrence of the disease is extremely rare in the same family, this would be performed because of the improbable possibility of germline mosaicism in one of the parents (Shankar et al., 2010). Also, pre-implantation genetic diagnosis may be available for families in which the disease-causing mutation has been identified in an affected family member.

3.8 Treatments: What has Been Done and Future Strategies

To date, no definitive therapy is proven to be effective for any of the progressive and deleterious aspects of HGPS. Patients are generally treated conservatively and following precautions and indications may improve their condition (Shankar et al., 2010). For example, low dose aspirin can help the cardiovascular signs, high-calorie dietary supplements can help in reducing weight loss, while physical therapy can help maintaining joint motion and muscle stretching and strengthening (Shankar et al., 2010; Ahmed et al., 2017).

Therapeutic approaches for HGPS treatment can be intended to work at different levels and several strategies have been adopted to correct defects of HGPS. We can broadly summarize the approaches in the following main ones: to decrease the toxicity of farnesylated prelamin, to block the cryptic splicing site leading to mature progerin mRNA production, to reverse the cellular phenotypes, or to degrade progerin or farnesylated prelamin A within cells (Cau et al., 2014; Gordon et al., 2014).

The discovery of the cause of HGPS in 2003 represented a key element in therapy strategies studies. Since the pathological phenotypes seen in HGPS arise from a farnesylated, mutant prelamin A, inhibition of its farnesylation was thought to reverse these phenotypes. In 2005, many publications reported that the blebs observed in the nuclear membranes of cultured fibroblasts from HGPS could be eliminated by treatment with farnesyltransferase inhibitors (FTIs), drugs originally developed to potentially block the activation of Ras proteins in different cancers (Capell et al., 2005; Glynn & Glover, 2005; Mallampalli et al., 2005; Toth et al., 2005; Yang et al., 2005). The importance of Ras proteins in cell proliferation, differentiation and survival is well known, as also the relation of overactive Ras signalling with cancer.

In 2006, Fong et al. demonstrated that FTIs ameliorated the pathology exhibited in a mouse model of progeria (*Zmpste24*$^{-/-}$*).* Other *in vivo* studies on animal models of progeria treated with FTIs reported that cardiovascular defects, bone mineralization, and weight were improved, and lifespan was extended with this therapy (Yang et al., 2006; Capell et al., 2008). Still, it has to be said that these mice models had a fairly severe disease phenotype and died prematurely (Yang et al., 2008) and that FTI gave better results in cultured cells from human patients than in mouse models of progeroid syndromes. Indeed, only a small percentage of progerin/prelamin A seems to be unprenylated in FTI-treated animals. Potentially, FTI treatment could negatively affect B-type lamin dynamics, necessary for the nervous system and not only (Adam et al., 2013; Stewart, 2014). However, the results of these studies were

considered promising and led to propose FTIs as a possible treatment for HGPS (Rusiñol & Sinensky, 2006). So, on May 7th 2007 the first ever clinical drug trial began. Twenty-six patients were treated with lonafarnib (LON) administered orally twice per day (150 mg/m^2) for a period of two years. Although control studies were not possible, LON appeared to be effective for progeria since every child showed improvement in one or more of four ways (i.e., gaining additional weight, better hearing, improved bone structure and/or flexibility of blood vessels) with some of the HGPS patients developing mild drug-related side effects (Gordon et al., 2012). The benefit of FTI for progeria is controversial since after treatment donut-shaped nuclei in cultured cells and in tissues from wild type mice or from human progerin still appears. This phenomenon is linked to an increase of the proteasomal degradation of pericentrin, a centrosomal protein (Verstraeten et al., 2011). Stop mutations in pericentrin lead to dwarfism (Rauch et al., 2008) and to abnormalities in ataxia telangiectasia and Rad3-related (ATR)-dependant DNA damage response (Griffith et al., 2008). FTI are known inhibitors of the histone deacetylase HDAC6 (Marcus et al., 2005; Zhous et al., 2009), whose main cytosolic targets are tubulin, tau and several other microtubule-associated proteins (Hubbert et al., 2002; Ding et al., 2008; Zilberman et al., 2009). Therefore, FTI-induced nuclear shape abnormalities could result from defects in both mitotic spindle microtubules and in centrosome organization and functions (Kovacs et al., 2004). After the beginning of the first clinical trial, it was demonstrated that an alternative prenylation pathway, called geranyl geranylation, may be activated in the presence of FTIs, offering possible explanations for the only moderate efficiency of the FTI treatment (Yang et al., 2008).

In 2007, Dr. Carlos Lopez-Otin, University of Oviedo (Spain), during a Progeria Research Foundation Scientific Workshop presented laboratory studies in which he demonstrated that zoledronic acid (ZO) and pravastin (PRA) administrated together improved disease in progeria cells and extended lifespan in different mouse models (Varela et al., 2008; PRF, 2017c). Pravastatin is a member of the drug class of statins and it is usually used for lowering cholesterol and preventing cardiovascular disease, while zoledronic acid is a bisphosphonate, usually used as a bone drug for improving osteoporosis, and to prevent skeletal fractures in people suffering from some forms of cancer (PRF, 2017c). They act as enzymes of the isoprenoid biosynthesis pathway, zoledronic acid inhibiting farnesyl-pyrophosphatase synthase and pravastatin inhibiting the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (Varela et al. 2008). This synergistic combination offers the advantage of blocking

both the farnesylation and geranyl-geranylation of progerin/prelamin A, avoiding the alternative isoprenylation by geranyl-geranyl transferase I induced by FTI, already reported for Ras. This therapy was tested on the *Zmpste24*$^{-/-}$ progeria mouse model in which the aging-like phenotypes improved, ameliorating growth retardation, loss of weight, lipodystrophy, hair loss and bone defects. Likewise, the longevity of these mice was substantially extended (Varela et al., 2008). So, in 2009 another small clinical trial started, and PRA and ZO were administered together with LON for 1 month in order to prove feasibility before moving to a larger efficacy trial. The drugs in this trial were well tolerated and a triple drug efficacy trial started. Comparisons of the results of such clinical trial with the ones of LON monotherapy treatment revealed a small additional bone mineral density benefit. However, osteoporosis is not a primary contributor to premature mortality in HGPS, so it is not clear that this represents a clinically relevant advance (Collins, 2016), even because no added cardiovascular benefit was noted with the addition of pravastatin and zoledronic acid (Gordon et al., 2016). Furthermore, this triple-drug regimen induced more donut-shaped nuclei than the treatment by FTI alone (Verstraeten et al., 2011), and increased plaque formation in the carotid and femoral arteries, as well as apparent acceleration of the extraskeletal calcification that are a feature of HGPS (Collins, 2016). To date, the three drugs are not used together in HGPS patients, but whether an FTI plus a statin (without the bisphosphonate) might provid benefits it is still unknown (Collins, 2016).

As previously said, another strategy is to block the cryptic splicing site leading to mature progerin mRNA production. For this purpose, morpholino antisense oligonucleotides have been demonstrated effective *in vitro* in fibroblasts from HGPS patients (Scaffidi & Misteli, 2005). Morpholinos are small modified oligonucleotides that can sterically block the cryptic splice site in exon 11 of progerin pre-mRNA resulting in a concentration-dependent decrease in progerin mRNA and protein levels, markedly reducing the accumulation of progerin and its associated nuclear defects (Scaffidi & Misteli, 2005; Osorio et al., 2011). The combined administration of two antisense oligonucleotides that block the aberrant splicing in *Lmna* caused by the c.1827C>T;p.Gly609Gly mutation reduced progerin amounts also *in vivo* (Osorio et al., 2011). In such study conducted by Osorio et al. (2011), the progerin expression resulted downregulated in the liver and in the kidneys, the treatment significantly lengthened the lifespan of *Lmna*$^{G609G/G609G}$ mice and ameliorated most of the phenotypical and molecular alterations (Osorio et al., 2011). These findings, together with the increasing evidence that the

use of oligonucleotides for correction of splicing defects, has growing therapeutic applications. The encouraging preliminary results initiated a set up of a new clinical trial that is currently under design (Cau et al., 2014), even though this novel strategy presents significant regulatory challenges (Collins, 2016).

Still, some research groups targeted cellular HGPS phenotypes. Resveratrol, a stilbenoid activator of SIRT1, is a deacetylase involved in many cellular processes and that is showed to enhance health span in rodents (Vidak & Foisner, 2016). Resveratrol was also studied in *Zmpste24*$^{-/-}$ mice in which it slowed down weight loss and significantly extended lifespan (Liu et al., 2012a). The mechanism of the beneficial effect of resveratol in HGPS mice is unsure, but it has been shown that in the presence of progerin or prelamin A, SIRT1 exhibits reduced association with the nuclear matrix and decreased deacetylase activity, leading to rapid depletion of adult stem cells in *Zmpste24*$^{-/-}$ mice (Liu et al., 2012a). However, another study conducted with an osteoblast and osteocyte-specific progerin-expressing mouse model (Schmidt et al., 2012) did not reveal a beneficial effect of resveratrol (Strandgren et al., 2015). Thus, given that resveratrol is a natural product and that other more potent STACs exist, more detailed studies are needed to find out the links between A-type lamins and SIRT (Stewart, 2014), and whether resveratrol is a potentially promising drug for HGPS treatment. Endisha et al. (2014) demonstrated that restoring SIRT6 expression, which is diminished in HGPS cells and in normal human fibroblast approaching replicative senescence, may partially give phenotypic improvements, impeding senescence and the formation of dysmorphic nuclei, but the mechanisms underlining these observations needs to be investigated.

Another approach for treatment of HGPS aims at reducing progerin protein levels. Proteasomal degradation and autophagy are the two major cellular mechanisms involved in removing misfolded, mutant or aggregated proteins. Although a detailed study on potential pathways involved in progerin degradation has not been done so far, several observations suggest that progerin may be removed by activating macroautophagy (Vidak & Foisner, 2016). Treatment with rapamycin upregulates autophagy and extends lifespan from yeast to mammals (Jung et al., 2010; Madeo et al., 2010; Johnson et al., 2013). Rapamycin is known for its anti-aging properties in mice (Arriola Apelo et al., 2016) and this validates the theory that finding the cure for progeria may also benefit the entire aging population. Rapamycin raised great interest in the scientific community for its properties such as powerful antibiotic, antiproliferative and immunosuppressant (Chang et al., 1991). Mammalian target of

rapamycin (mTOR) is a protein kinase that controls cell growth, proliferation, and survival (Ballou & Lin, 2008). As a comment, it has been suggested that FTIs may indirectly affect mTOR by inhibiting the farnesylation of Rheb GTPase, an upstream activator of mTOR (Hanker et al., 2010). mTOR inhibition by rapamycin or its analogues may also mimic calorie restriction (CR) which is shown to counteract the onset of age-related diseases (Evangelisti et al., 2016). Moreover, it was hypothesized that mTOR inhibitors may reduce the age-associated inflammation, thus slowing down the progression of aging-related pathologies in humans (**Fig. 3.16**) (Evangelisti et al., 2016). Experimental studies demonstrated that rapamycin decreases the amount of progerin by 50%, improves the abnormal nuclear shape, extends the lifespan of progeria cells, and leads to autophagic degradation of toxic farnesylated prelamin A and progerin (Cao et al., 2011; Cenni et al., 2011; Graziotto et al., 2012). As a result of all the aforementioned findings, on April 2016 a new two-drug clinical trial started assessing everolimus in addition to lonafarnib. The first stage of the study will determine the safest maximum dose of everolimus in progeria children, then, if toxicity is manageable, the efficacy of its combination with LON will be evaluated (Collins, 2016). Everolimus is a semisynthetic rapamycin analogue (rapalogs), which has an improved oral bioavailability compared to rapamycin and is approved by the FDA (Evangelisti et al., 2016).

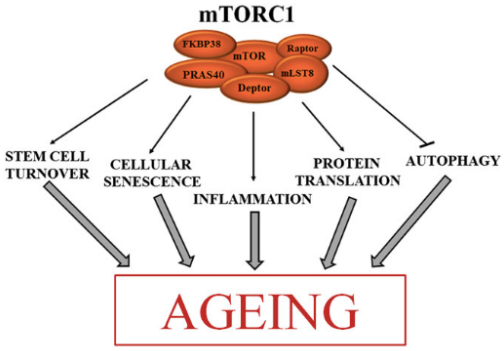


Fig. 3.16: *The mammalian target of rapamycin (mTOR) complex (mTORC1) signalling pathway contributes to aging through various cellular processes. Active mTORC1 activates stem cell turnover, cellular senescence and protein translation (arrows), while it inhibits autophagy. These conditions contribute to the aging of an organism (Evangelisti et al., 2016; reproduction licensed).*

Other possible therapeutic agents include tyrosine-kinase inhibitors (blocking fibrosis via TGFβ signalling), again statins (activating the peroxisome proliferator-activated receptor gamma - PPARγ - for adipogenesis) (Prokocimer et al., 2013), and also targeting the insulin-IGF1 signalling pathway has been investigated in a progeroid mouse model (Mariño et al., 2010; Cau et al., 2014).

In another recent study Vitamin D/Vitamin D Receptor (VDR) axis emerges as a new target for treatment of HGPS and potentially other lamin related diseases that show VDR deficiency and genomic instability (Kreienkamp et al., 2016). Because progerin expression increases with age, maintaining vitamin D/VDR signalling could keep the levels of progerin in check during

physiological aging. In fact, it has been demonstrated that HGPS cells reduce expression of VDR and DNA repair factors breast cancer gene 1 (BRCA1) and p53-binding protein 1 (53BP1) with progerin accumulation. Moreover, reconstituting VDR signalling via 1α,25-dihydroxyvitamin D3 (1,25D) treatment improves HGPS phenotypes, including nuclear morphological abnormalities, DNA repair defects, and premature senescence (Kreienkamp et al., 2016).

Inorganic pyrophosphate (PPi) treatment *in vivo* using *Lmna*^{G609G} mice was demonstrated to ameliorate excessive vascular calcification caused by reduced extracellular accumulation of pyrophosphate that results from increased tissue-nonspecific alkaline phosphatase activity and diminished ATP availability caused by mitochondrial dysfunction in vascular smooth muscle cells (Villa-Bellosta et al., 2013). Future studies in HGPS mouse models are warranted to investigate whether treatment with PPi in combination with alkaline phosphatase or PHOSPHO1 inhibitors and FTI and statins is more beneficial than current strategies (Villa-Bellosta et al., 2013).

Other potential therapeutic agents include scavengers of reactive oxygen species (ROS). ROS are bioproducts of cellular metabolism that damage DNA bases and block the progression of replication, and are linked to physiological aging (Zhang et al., 2014). N-acetyl cysteine reduced the amount of unrepairable DNA damage caused by the increased generation of ROS (Pekovic et al., 2011; Richards et al., 2011; Lattanzi et al., 2012; Sieprath et al., 2012). Also, methylene blue, a mitochondrial-targeting antioxidant was studied (Gonzalo et al., 2017). Sulforaphane, an antioxidant derived from cruciferous vegetables, was observed to stimulate proteasome activity and autophagy in normal and HGPS fibroblast cultures, reversing the cellular hallmarks of HGPS and representing a promising therapeutic avenue (Gabriel et al. 2015). Remodelin, is an inhibitor of N-acetyltransferase-10 able to rescue nuclear morphological abnormalities and proliferation defects, increase chromatin compaction and ameliorate the accumulation of DNA damage characteristic of progerin-expressing cells (Larrieu et al., 2014). Ongoing studies are monitoring the effect of remodelin on gene expression and evaluating its potential as a therapeutic strategy by using mouse models of progeria (Gonzalo et al., 2017).

Another set of promising compounds are the retinoids (Swift et al., 2013; Kubben et al., 2016). It was recently shown that the *Lmna* gene promoter contains retinoic acid responsive elements, and that treatment with all-trans retinoic acid (ATRA) results in downregulation of

Lmna gene expression. In HGPS patient-derived fibroblasts, ATRA treatment reduces significantly progerin expression and was more effective than rapamycin. Interestingly, ATRA synergized with rapamycin in downregulating progerin levels, which in turn ameliorated a variety of progerin-induced phenotypes (Pellegrini et al., 2015). These data are particularly promising because the low doses of the drugs required for the combined treatment avoid the potential side effects associated with chronic treatment. Retinoids were also identified in a high-throughput, high-content based screening of a library of FDA approved drugs as a class of compounds able to revert cellular HGPS phenotypes (Kubben et al., 2016). These findings stress the importance of testing *in vivo* the efficacy of retinoids in ameliorating HGPS defects without inducing toxicity.

Overall, these findings together suggest that compounds acting by decreasing progerin levels in the cell could represent a potent tool for new treatments. Autophagy-activating drugs could be particularly beneficial in progeria treatment, but prudent *in vivo* analyses have to be conducted before including them in clinical trials (Vidak & Foisner, 2016).

The generation of HGPS-derived induced pluripotent stem cells (iPSCs) were reported for the first time by Zhang et al. (2011) providing a powerful new tool to unravel the molecular and physiological mechanisms of premature and normal aging (Misteli, 2011). Moreover, the HGPS-iPSCs, and their derivatives, are also useful for drug discovery (Misteli, 2011). iPSCs described by Zhang et al. (2011) are able to differentiate into five lineages, including vascular smooth muscle cells (VSMCs) and mesenchymal stem cells (MSCs), confirming their multipotency. These cells now offer a useful experimental system to probe the effect of progerin on the differentiation of various cell lineages, something that could not be done before because of the inability to obtain tissue samples from patients. These cells also opened the door to performing critical experiments, such as transplantation of HGPS-derived MSCs into the vasculature of animal models to probe the physiological mechanisms that participate in the vascular defects experienced by HGPS patients (Zhang et al., 2011).

Lee et al. (2016) published a research article in which administration of JH4 (from the Janus kinase (JAK) family of tyrosine kinases) to $Lmna^{G609G/G609G}$-mutant mice, resulted in a marked improvement of several progeria phenotypes and in an extended lifespan. Treated $Lmna^{G609G/G609G}$ mice survived up to more than 25 weeks, much more compared to controls (20 weeks of age), and this effect was even more obvious in $Lmna^{G609G/+}$. JH4 is able to bind to progerin and block its interaction with lamin A/C. Being selective for progerin, there would be

very minimal and nonspecific side effects. Therefore, it was proposed as a new treatment strategy for HGPS and, eventually, for age-associated alterations involving nuclear envelope abnormalities (Lee et al., 2016).

Lastly, there is increasing knowledge that during aging of mammalian cells/tissues, functions of many cytokines become abnormal and this leads to low-grade chronic inflammation, known as "inflammaging", causing various age-related diseases (Puzianowska-Kuźnicka et al., 2016; Rath, 2017). It has been reported that a balance between the pro-inflammatory and the anti-inflammatory cytokines would lead to adaptive aging delaying or escaping the diseases and resulting into healthy longevity. A breakdown of this balance, due to over-production of the pro-inflammatory cytokines, would result in accelerated aging, frailty, age-related diseases and reduced life-expectancy (Minciullo et al., 2016). The state of low-grade, persistent and chronic inflammation also leads to generation of excessive oxidative stress damaging cellular macromolecules, sub-cellular structures and disease phenotypes (Rath, 2017). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a protein complex that controls transcription of DNA, cytokine production and cell survival, and it is involved in cellular responses to stimuli such as stress, cytokines, ultraviolet irradiation, heavy metals, free radicals, oxidized LDL, and viral or bacteria antigens. NF-kB hyperactivation has been related to the aging process (Adler et al. 2007), and is well documented in numerous age-associated diseases. NF-kB hyperactivation, together with the secretion of high levels of pro-inflammatory cytokines, was demonstrated in two different mouse models of accelerated aging (*Zmpste*$^{24-/-}$ and *Lmna*$^{G609G/G609G}$ mice) (Osorio et al., 2012). In such study, inhibition of NF-kB signalling was obtained in *Zmpste*$^{24-/-}$ mice thanks to a genetic technique based on the use of *RelA*-haploinsufficient mice (*RelA*$^{+/-}$); in *Lmna*$^{G609G/G609G}$ mice inhibition was obtained pharmacologically using sodium salicylate. The inhibition of NF-kB signalling prevented the age-associated features typical of the models and extended their longevity (Osorio et al., 2012). As it can be seen in **Fig. 3.17**, *Lmna*$^{G609G/G609G}$ mice had high serum levels of IL-6, which were linked to a systemic inflammation condition and immunological alterations (Osorio et al., 2012). The authors proposed that among the plethora of pro-inflammatory cytokines secreted by senescent cells, IL-6 together with chemokine (C-X-C motif) ligand 1 (CXCL1) and TNF-a, might have essential roles in progeria development by nonautonomous stimulation of surrounding cells through the activation of their cognate cell surface receptors and signal transduction pathways (Coppe et al., 2010; Freund et al., 2010).

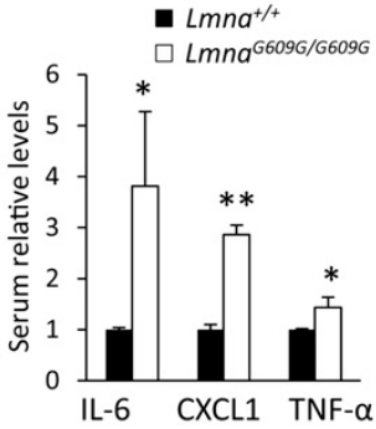


***Fig. 3.17**: Serum determinations of IL-6, CXCL1, and TNF-a in 3-month-old Lmna$^{+/+}$ (n = 5) and Lmna$^{G609G/G609G}$ (n = 5) mice. Plot represents relative mean values 6 SEM. (*) P < 0.05; (**) P < 0.01, two-tailed Student's t-test (Osorio et al., 2012; reproduction licensed).*

Interleukin 6 (IL-6) is a well-known cytokine, involved in a variety of processes such as development and differentiation, and promotes inflammation (Ershler, 1993). IL-6 dysregulation is demonstrated to be a direct cause of physiologic decline with aging (Gomez et al., 2010; Puzianowska-Kuźnicka et al., 2016) and modulation of its production or effects could offer a major breakthrough in prevention and treatment of people at advanced old age (Maggio et al., 2006). In accordance to the current knowledge, acting against low grade inflammation and in particular inhibiting IL-6 may be beneficial in aging related disorders and thus, also in progeria patients.

In conclusion, all these *in vitro* and *in vivo* animal studies, together with the results of the first clinical trials, show that it is possible to achieve improvements of the disease phenotypes. Also, the increasing knowledge on biological mechanisms involved in aging can help in finding new therapeutic strategies to improve HGPS patient's conditions and also of the older people. However, for what concerns HGPS, we still need to better understand the underlying disease mechanisms to be able to tackle specific aspects of the disease in a more focused approach. It will also be important to elucidate which of the numerous pathways found to be impaired in HGPS are the most relevant.

3.9 Animal Models for HGPS

Recent advances in defining molecular pathways implicated in aging have been obtained by the use of genetically tractable model systems, ranging from yeast, to flies and nematodes, to mice (Gurkar & Niedernhofer, 2015). Also, several animal models have been developed to study laminopathies. In particular for laminopathies, the most studied animals are the mouse, *C. elegans* and *Drosophila.* These last two are attractive especially for studying the basic functions of nuclear lamina genes and for understanding the mechanisms behind laminopathic diseases (Prokocimer et al., 2009). On the other hand, the mouse is interesting for life- and healthspan studies due to its relatively short lifespan and low cost of housing, genetic similarity to humans, genetic tractability, and the large amount of available baseline phenotypic data (Gurkar & Niedernhofer, 2015; Kõks et al., 2016). The development of animal models that phenocopy segmental progeroid syndromes, such as HGPS, are particularly interesting because not only they can provide experimental systems useful to investigate the basis of particular pathologies associated with aging, but they can also be used to perform preclinical testing of therapeutic strategies against these alterations (Osorio et al., 2009; Burtner & Kennedy, 2010). Genome maintenance mechanisms are generally highly conserved between species and for this reason mice defective in such pathways are important models (Gurkar & Niedernhofer, 2015). Since the discovery of the cause of HGPS many progeroid mouse models have been created, allowing the recent development of the first therapeutic approaches for this disease.

It is important to keep into consideration that mice and humans may show different features or sensitivity to progeroid-causing alterations, and these differences have to be carefully understood to interpret results derived from the use of murine models (Osorio et al., 2009; Kõks et al., 2016). These differences should be interpreted with even more caution when translated to normal old mice and humans.

Zmpste24-deficient mice is one of the existing animal models of accelerated aging, created in 2002 and still in use in biomedical research. As previously described, *Zmpste24* is the metalloproteinase involved in the post-translational maturation of prelamin A, and the result of its missing is the expression of farnesylated prelamin A which produces various progeroid phenotypes. At 4–6 weeks of age *Zmpste24*$^{-/-}$ mice start displaying growth retardation, alopecia, loss of adipose tissue, multiple spontaneous bone fractures, abnormal nuclear

morphology and premature death, as well as muscular dystrophy and dilated cardiomyopathy (**Fig. 3.18**) (Bergo et al., 2002; Pendas et al., 2002). Only the year after the creation of this model, when the molecular cause of HGPS was discovered, the resemblance of these mice phenotype to the clinical features characteristic of HGPS were explained by the proteolytic defect (Osorio et al., 2009). A few years later, the first knock-in *Lmna*^{HG} mice was engineered. This mice model yields exclusively progerin but no wild-type lamin A and lamin C, displaying phenotypes similar to HGPS children including alopecia, loss of subcutaneous fat, osteoporosis and premature death, but without cardiovascular defects (Yang et al., 2005). This model presented also other problems, such as difficulty in the maintenance of the model due to fertility issues of the heterozygous, and differences in the expression levels of progerin and the ratio progerin/normal lamin A/C compared to HGPS patients. Furthermore, the different genomic base of the model compared to the human disease doesn't make this model suitable to test specific target drugs (Osorio et al., 2009). In contrast, a transgenic C57BL/6 mouse model that carries the mutated G608G human *Lmna* allele on a bacterial artificial chromosome (G608G^{BAC}) develops progressive loss of vascular smooth muscle cells (VSMCs), which constitute the most common cause of death in HGPS patients, but did not show most of the other pathologies limiting its utility as a model of accelerating aging (Varga et al., 2006). Also, a mouse model exclusively expressing the non-farnesylated form of progerin (*Lmna*^{nHG}) was generated showing milder abnormalities and extended longevity compared to *Lmna*^{HG} mice (Yang et al., 2008). This amelioration is comparable to that obtained in *Zmpste24*$^{-}$ deficient animals treated with a combination of statins and aminobisphosphonates capable of blocking efficiently prelamin A prenylation (Varela et al., 2008). Since accumulation of non-farnesylated progerin also causes aging signs, blocking only progerin production might not be sufficient in reaching a complete efficacy (Yang et al., 2008). Davies et al. (2009), described a *Lmna* knock-in allele encoding a geranylgeranylated progerin that causes a progeroid phenotype, providing a formal demonstration of the toxicity of this alternatively modified protein (Osorio et al., 2009).

Fig. 3.18: *Photograph of two littermate progeny of a Zmpste24 heterozygote cross, at 3 months of age (Pendas et al., 2002; reproduction licensed).*

Another mouse model, in which a point mutation in *Lmna* caused loss of exon 9 (*Lmna*$^{L530P/L530P}$), also displayed phenotypes overlapping with HGPS (Mounkes et al., 2003; Hernandez et al., 2010) such as loss of subcutaneous fat, decreased bone density, osteoporosis, abnormal dentition, thin hyperkeratotic skin, growth retardation and death by 3-4 weeks of age (**Fig. 3.19**). The mechanism is still unclear and appears to be linked to the reduced expression of many genes encoding extracellular matrix (ECM) components, thus it may differ from that of the classical HGPS (Mounkes et al., 2003; Stewart, 2014).

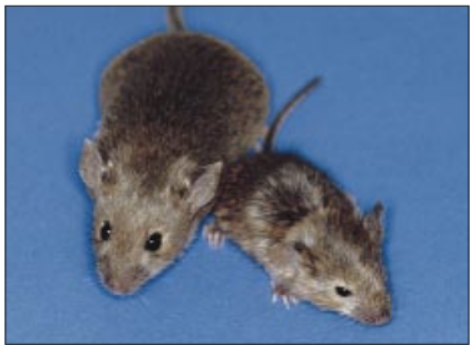


***Fig. 3.19**: Lmna*$^{L530P/L530P}$ *homozygous mice exhibit severely retarded growth and die early. The smaller Lmna*$^{L530P/L530P}$ *mouse weighed 5.37 g; the littermate (21.88 g) was wild type (Mounkes et al., 2003; reproduction licensed).*

All the above-mentioned mice models have a C57BL/6 background (Bergo et al., 2002; Pendas et al., 2002; Mounkes et al., 2003; Yang et al., 2005; Varga et al., 2006; Yang et al., 2008; Davies et al., 2009).

Finally, transgenic mouse models with tissue-specific progerin expression have been generated in addition to those expressing progerin ubiquitously (Sagelius et al., 2008; Rosengardten et al., 2011; Schmidt et al., 2012; Baek et al., 2015). However, their usefulness is limited when investigating therapies that should have an impact on all aspects of the disease.

As we can see, several knock-out, knock-in and transgenic animal models have been used to provide valuable information on the role of alterations in the prelamin A post-translational maturation in accelerated aging and have been used to develop therapeutic strategies against progeroid symptoms (Osorio et al., 2009). However, all these mouse models described above only partially phenocopy HGPS patients and until 2011 new animal models were required to test *in vivo* anti-progeria therapies. Furthermore, none of the described mice were appropriate to test approaches such as those targeting the alternative splicing responsible for progerin production. This problem was addressed by Osorio et al. (2011), who created a knock-in mouse strain that carries a HGPS mutation in the mouse *Lmna* gene (*Lmna* ^{G609G}; 1827C>T; Gly609Gly; **Fig. 3.20**). Mice that carry the *Lmna* ^{G609G} allele express lamin C, lamin A, and progerin (due to abnormal splicing of the endogenous *Lmna* mRNA), reproduce the same molecular situation present in HGPS patients and phenocopy the main clinical manifestations

of the disease, including shortened life span, bone disease and cardiovascular aberrations (Osorio et al., 2011). This model has been used since its creation to test possible therapies for progeria patients (Osorio et al., 2011; Osorio et al., 2012; Villa-Bellosta et al., 2013; Lee et al., 2016), and is considered to be the best animal model for preclinical drug testing not only for HGPS but also for normal aging where progerin accumulation is associated to the age pathologies (Osorio et al., 2012).

Despite these considerations, no information on the housing and breeding of this model is available in literature, and the clinical manifestations of homozygous and heterozygous need to be studied and described more in detail and in a larger colony.

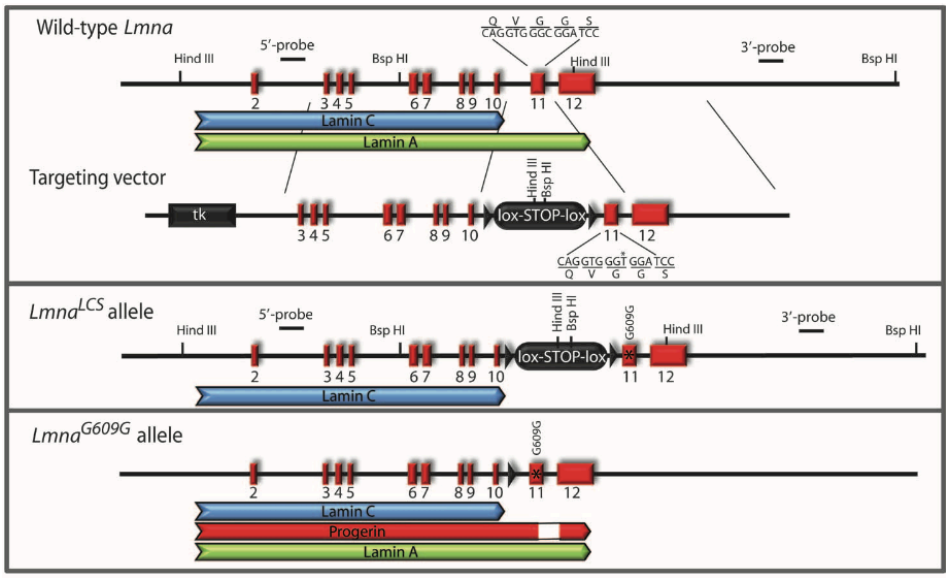


Fig. 3.20: *Generation of knock-in Lmna^{G609G} mice. Schematic representation of the wild-type Lmna locus, targeting vector, and targeted allele. Positions of restriction enzyme cleavage sites and probes used for Southern blot analysis are shown (Osorio et al., 2011; reproduction licensed).*

Chapter 4

The Study of the *Lmna*^{G609G} Transgenic Mice Phenotype

4.1 Objective

The overall objectives of this study were to characterize the *Lmna*$^{G609G/G609G}$ and *Lmna*$^{G609G/+}$ phenotypes, and to describe the colony in a more detailed way compared to previous studies. In this study, many features of a large *Lmna*^{G609G} colony were observed for a 2-years period. We gave a first description of the breeding and housing conditions and tried to underline the possible interference of the genotypic background with the phenotype expression.

The final aim was to gather data that can help researchers using *Lmna*^{G609G} mice for their investigations, in order to optimize the breeding and housing conditions, to thoroughly understand similarities and differences between the mouse model and human patients, pointing out confounding effects that could derive from the well known genetic background of C57BL/6.

This study was conducted in collaboration with the National Research Council, Institute of Molecular Genetics - Unit of Bologna, Italy.

4.2 Materials and Methods

All *in vivo* studies were performed at the Laboratory Animal facility of the Department of Veterinary Medical Sciences (DIMEVET), Alma Mater Studiorum – University of Bologna, with the authorization of the experimental protocol by the Italian Ministry in compliance with the Legislative Decree 26/2014 (Protocol number 653/2016-PR released on July 7th, 2016). An integration to the treatment protocol was prepared in December 2017.

Furthermore, experimental procedures were carried out following Standard Operating Procedures (SOP). The three main SOPs followed were:

1) SOP 7.5-02-01: Animal house management

2) SOP 7.5-02-02: Conditions to be applied to the animal house locals

3) SOP 7.5-02-03: Safeguard of the animals in the animal house.

4.2.1 HGPS Mouse Model

The HGPS transgenic mice were provided by Professor Carlos-Lopez Otín (University of Oviedo, Spain, Departamento de Bioquímica). Briefly, to generate the knock-in mouse strain carrying the HGPS mutation, the wild-type mouse *Lmna* gene was replaced with a mutant allele that carried the c.1827C>T;p.Gly609Gly mutation, which is equivalent to the HGPS c.1824C>T;p.Gly608Gly mutation in the human *Lmna* gene. The knock-in mice were produced with embryonic stem cells derived from a 129/OLA, microinjected to C57BL/6 blastocysts to produce chimeric mice that were then strain backcrossed to C57BL/6. Osorio et al. (2011) provided a detailed description on how the mouse model was created together with a first description of the model. Mice that carry the *Lmna*^{G609G} allele express lamin C, lamin A, and progerin (due to abnormal splicing of the endogenous *Lmna* mRNA), reproducing the same molecular situation present in HGPS patients (**Fig. 4.1**).

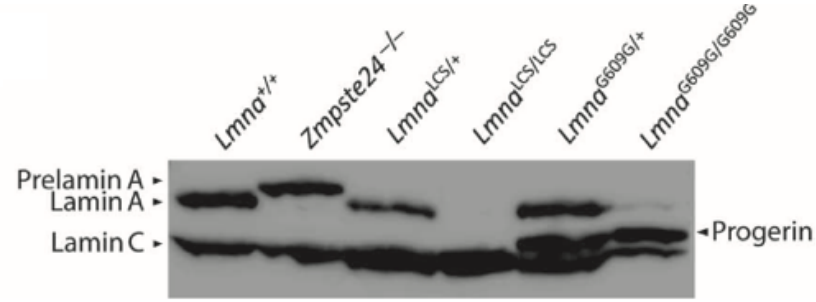


Fig. 4.1*: Western (immuno) blot analysis of mouse adult fibroblasts obtained from the mice with the various genotypes used in the study of Osorio et al., 2011. Lamin A, lamin C, prelamin A, and progerin were detected with a monoclonal antibody against lamin A/C (Manlac-1) (Osorio et al., 2011; reproduction licensed).*

4.2.2 Housing and Breeding

Mouse breeding was conducted in the Laboratory Animal facility of Department of Veterinary Medical Sciences (DIMEVET), Alma Mater Studiorum – University of Bologna, starting from three heterozygous (*Lmna*$^{G609G/+}$) progenitors (one male and two females) provided by Professor Otín, University of Oviedo (Spain). At the time of arrival, mice were two-month old and were kept in quarantine for acclimatization for 10 days.

Mice were maintained under a 12 hours of dark/light life cycle (200 lux at the cage level) in an environment controlled for temperature (20-24°C) and humidity (40-70% relative humidity). Temperature and humidity were daily recorded via specific data logger, subjected to calibration as provided by DIMEVET Plan Quality for the calibration of instruments and facilities.

The mice were housed with the same sex littermates in conventional polycarbonate cages, in accordance with the indications of the Italian Decree 26/2014 (Mouse Cage 1284L001, 365 mm L × 207 mm W × 140 mm H, Tecniplast, Varese, Italy). Breeding pairs were housed in bigger cages (Mouse Cage 1291H001, 425 mm L × 266 mm W × 185 mm H, Tecniplast, Varese, Italy).

Litter (Lignocel, Hygienic Animal Bedding) and the environmental enrichment (e.g. cardboard rolls, sizzle nest) guaranteed to the animals the opportunity to carry out the typical behavior of the species.

The mice received standard chow (Teklad 20/18 Rodent Diet, Envigo, Udine, Italy) and water *ad libitum*. Animals ate while fully inverted by grasping onto the wire cage top. A subset of these mice (cage 1 to 30 - G1-G30), were fed also with moistened chow deposited on aluminium foils on the bottom of the cage starting approximately from the age of 10 weeks, in order to assure feed intake and weight maintenance to the less motile ones.

Breeding pairs were composed of 2 females for each male starting from the age of 1-2 month until the age of 5-7 month. Since the mutation carried by these mice leads to premature aging, we hypothesize that *Lmna*$^{G609G/+}$ has a shorter reproductive life than wild type (*Lmna*$^{+/+}$) and they were retired before 1 year of age, differently from what suggested for this strain by Baumans (2007). To increase the probability of having *Lmna*$^{G609G/+}$ and *Lmna*$^{G609G/G609G}$ mice instead of *Lmna*$^{+/+}$, we favorited intercross breeding female *Lmna*$^{G609G/+}$ × male *Lmna*$^{G609G/+}$. However, in order to assure the maintenance of the strain we also coupled female *Lmna*$^{+/+}$ × male *Lmna*$^{G609G/+}$. According to Mendelian expectations, *Lmna*$^{G609G/+}$ x *Lmna*$^{G609G/+}$ generate

50% *Lmna*$^{G609G/+}$, 25% *Lmna*$^{+/+}$, and 25% *Lmna*$^{G609G/G609G}$, while *Lmna*$^{+/+}$ x *Lmna*$^{G609G/+}$ generate 50% *Lmna*$^{+/+}$ and 50% *Lmna*$^{G609G/+}$. It is important to underline that only the homozygous and heterozygous mice develop the disease, the first in a more severe way than the latter; wild type mice were used as controls.

To assess if *Lmna*$^{G609G/G609G}$ were sterile or not, we bred two females *Lmna*$^{G609G/G609G}$ with one wild type male. Also, one male *Lmna*$^{G609G/G609G}$ was caged with two heterozygous females.

Pups were kept with dams until an age of ~30 days in order to improve survival especially of the smaller ones (i.e., homozygous and heterozygous). During the weaning, they were identified via headset marking (**Fig. 4.2**), and the tissue clipped with this procedure, together with the tail tip, were used for the genotyping. This procedure was always conducted in deep general anesthesia (2.5% isoflurane in O_2) using an anesthetic machine (Surgivet, Smith Medical Vet Division, Isotec 4 + LFY-1-a Medical Oxygen Concentrator, Biological Instruments, Besozzo, Varese, Italy). For the mouse identification, the letters L (for littermate) and G (indicating the number of the cage) were used and mice were numbered according to the marking on the ear (for example, "L1G1" i.e.: littermate number 1 in the cage number 1). The cages were distinguished by means of card on which date of birth, species, sex, genotype, parents, identification number of each animal, and eventually treatment, had been noted.

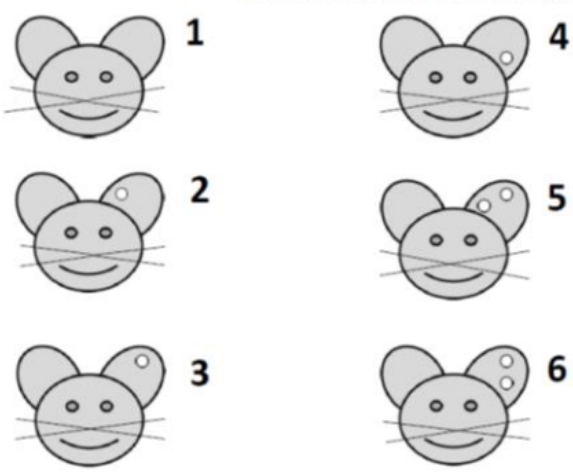


Fig. 4.2: *Headset marking. Based on the position of the left ear hole each mouse was identified by a number following the letter L.*

4.2.3 Genotyping

Genotyping was carried out at the Laboratory of Medical Genetics, Fondazione Policlinico Torre Vergata, Rome (Dr Maria Rosaria D'Apice). Genomic DNA was extracted from a few mm^3

of skin sample of the terminal, not ossified, part of the tail tip obtained during the headset marking, and from the ear tissue deriving from such procedure.

4.2.4 Colony Health Surveillance and Animal Monitoring

The mice colony was maintained in a conventional facility. Animal's health status was monitored by exposing sentinels to the dirty bedding, water bottle and food from all the cages of the colony. The sentinels underwent the Federation for Laboratory Animal Science Associations (Felasa) annual complete profile monitoring, and testing was performed by Envigo (http://www.envigo.com). The investigation included the most common viruses, bacteria, mycoplasma, fungi and ecto- and endoparasites in laboratory mice. Protective personal equipment (PPE) was always used in order to limit contamination between humans and mice.

The overall assessment of the health and welfare of the mice was conducted daily through an evaluation of the animals in their home cage. A hands-on exam was conducted twice weekly. During this exam mice were restrained on the top of the cage and felt with a finger running over the animal's coat to feel for wounds or masses, and the hydration level was assessed (Burkholder et al., 2012). Since the animals did not have conditions such as tumours that can cause increase in body weight while breaking down fat and muscle, we preferred assessing health through weighing the mice twice weekly instead of using the body condition score method (Ullman-Culleré & Foltz, 1999). The animals were weighted using a calibrated scale (Kern 440-47N, Kern & Sohn GmbH, Balingen, Germany) with a plastic container in which the mouse was placed (**Fig. 4.3**). Mice in reproduction were not weighted, and were only checked from the outside of the cage or during litter change and at weaning of the pups, in order to reduce stress connected to manipulation which could negatively influence reproduction. All observations were recorded on a dedicated register.

***Fig. 4.3**: Mouse in a plastic container on a calibrated scale.*

4.2.5 Grip Strength Test, Open Field Test, and Numerical Scoring System Specific for *Lmna*^{G609G} Transgenic Mice

Grip strength was evaluated using the inverted grid test (Brooks & Dunnett, 2009) for a subset of mice as described in other studies using HGPS mice models (Bergo et al., 2002; Fong et al., 2004; Yang et al., 2006). Briefly, 5 males for each genotype were observed for 60 seconds at 1, 2, 3 months of age, and for heterozygous and wild type also at 6 and 9 months of age, in order to test the ability of the mouse to remain clinging to the cage lid after it was turned upside down. The grid was slightly agitated before inverting it to increase animals grip. The test was repeated up to 3 times if animal did not succeed remaining attach to the cage lid for the 60 seconds of observation.

The open field test (OFT) is widely used to measure locomotor activity and anxiety-like behaviour (Prut & Belzung, 2003). A subset of mice underwent the OFT at 1, 2, 3, 6 and 9 months of age. A semi-transparent plastic white rectangular box (≈ 70 x 50 cm), was marked from underneath the surface with parallel lines both horizontal and vertical, forming a grid of 35 total quadrants, each measuring ≈ 9 x 9 cm (**Fig. 4.4**). With another indelible green marker, a central area of ≈ 39 x 23 cm was delimited. The arena was placed at the center of the room and illuminated at about 200 lux. At the beginning of the test, each mouse was set in the middle of the arena and always the same two experienced operators during a 5 minutes period observed and recorded manually: travelled quadrants (Q), time spent moving (TM), time spent in the central area (TC), vertical movements (VM), freezing (F), grooming (G), feces (C) and urines (U). The operators were positioned as far as possible from the arena and remained still and quiet throughout each trial. The room was isolated from sound and unintentional interruptions were firmly avoided.

***Fig. 4.4**: OFT arena. Total arena (≈ 70 x 50 cm) divided in 35 quadrants (≈ 9 x 9 cm each). The central area (≈ 39 x 23) was delimited by the green marker.*

A spray bottle with 30% alcohol was used before and after each OFT for a general wipe down of the arena. Each mouse was placed in a new cage rather than back with the cage mates, as reintroduction of the mouse may modify behaviour of mice not yet tested (Gould et al., 2009). Independently from the OFT, a numerical scoring system (NSS) specific for *Lmna*^{G609G} transgenic mice was used to numerically describe mice conditions and to compare the different genotypes at the same time intervals. This scoring system was used at the end of each OFT. In particular, the parameters considered and the attributed scores are shown in **Table 4.1**.

Parameter	Animal Condition	Score
Fur	Shiny, thick, black	1
	Still in good condition, however slightly jagged and streaked with grey	2
	Beginning of the periocular alopecia, jagged, streaked, opaque and slightly dirty fur	3
	Periocular alopecia, thin, jagged, streaked, opaque and dirty fur	4
Gait analysis	Normal	1
	Unstable/duck walk	2
Activity	Active, lively, curious, jumps, runs, digs the litter	1
	Active, lively, does not jump, alternates time spent moving and time spent still	2
	Active but not particularly lively, moves slowly	3
	Tends to stay still, trembles and is hypoactive	4
Total Score		

***Table 4.1**: Numerical scoring system (NSS) specific for Lmna^{G609G} transgenic mice. Grades were given always by the same operator. 3= best condition possible; 10= worst condition possible.*

4.2.6 Radiological Examinations

Some of the mice were subjected to X-ray examination of the skull and spinal cord. Radiographic examinations and the values used to obtain good quality images are shown in **Table 4.2**.

The total body X-ray examination of the animal sedated by protocol (2.5% isoflurane in O_2 using the anaesthesia machine), was performed in a single lateral view with the right decubitus. Care was taken to avoid overextension or flexion of limbs. Radiographs of mice not properly positioned, or that were under- or overexposed were excluded from the analysis.

Equipment employed for the X-rays	Remote controlled Mecall SUPERIX and methodical CR, Computed Radiology (FCR Capsula Fujifilm)
kV	50
mAs	2 (80 mA and 2.5ms)

***Table 4.2**: X-rays equipment and values used in order to achieve good quality images.*

Radiographic studies were evaluated by a single operator and performed with the collaboration of the Service of Diagnostic for Images in DIMEVET.

In particular, progression of spinal deformity was evaluated by using the kyphosis index (KI) as described by Laws & Hoey (2004). Briefly, KI was calculated from a line drawn between the caudal margin of the last cervical vertebra to the caudal margin of the sixth lumbar vertebra (which usually corresponds to the cranial border of the wing of the ilium) divided by a line perpendicular to this from the dorsal edge of the vertebra at the point of greatest curvature (**Fig. 4.5**).

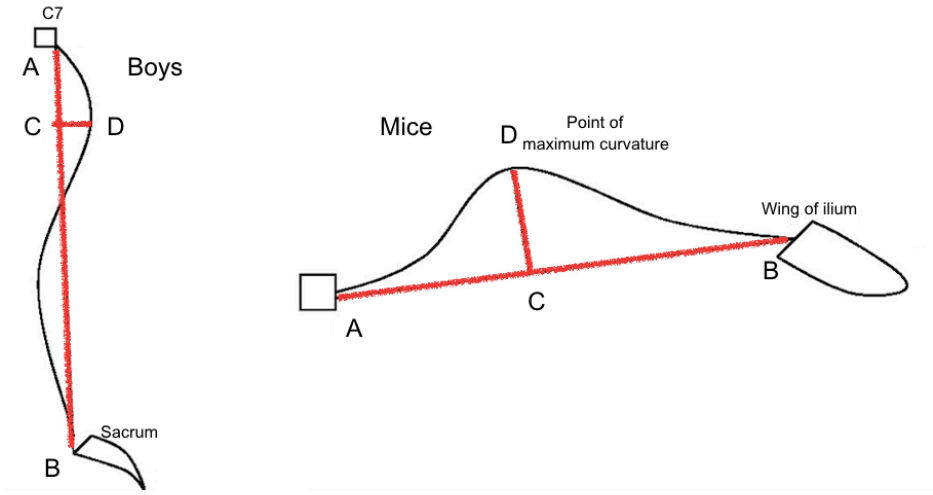


***Fig. 4.5**: Kyphosis index (KI). KI was calculated from radiographs of anesthetised mice positioned in right lateral recumbency. KI = AB/CD (adapted from Laws and Hoey, 2004)*

4.2.7 Anaesthesia

Isoflurane is a particularly suitable volatile liquid for anaesthesia. It is characterized by a wide safety margin and excreted by exhalation. Liver metabolism and different excretion routes are not significant. Gaseous agents, generally, have the distinct advantage of enabling a rapid recovery from anaesthesia, so the animal regains its homeostasis quickly (e.g. posture, temperature regulation) (Kaliste, 2007).

Mice were placed in an induction box or chamber of the anaesthetic machine (Surgivet, Smith Medical Vet Division, Isotec 4 + LFY-1-a Medical Oxygen Concentrator, Biological Instruments, Besozzo, Varese, Italy) in the condition of 2.5% isoflurane in O_2, and then, if needed, anaesthesia was maintained always with isoflurane using a face mask (**Fig. 4.6**).

Anaesthesia was used during X-rays and at weaning for both the headset marking and to gather the tail tip in order to genotype the mice.

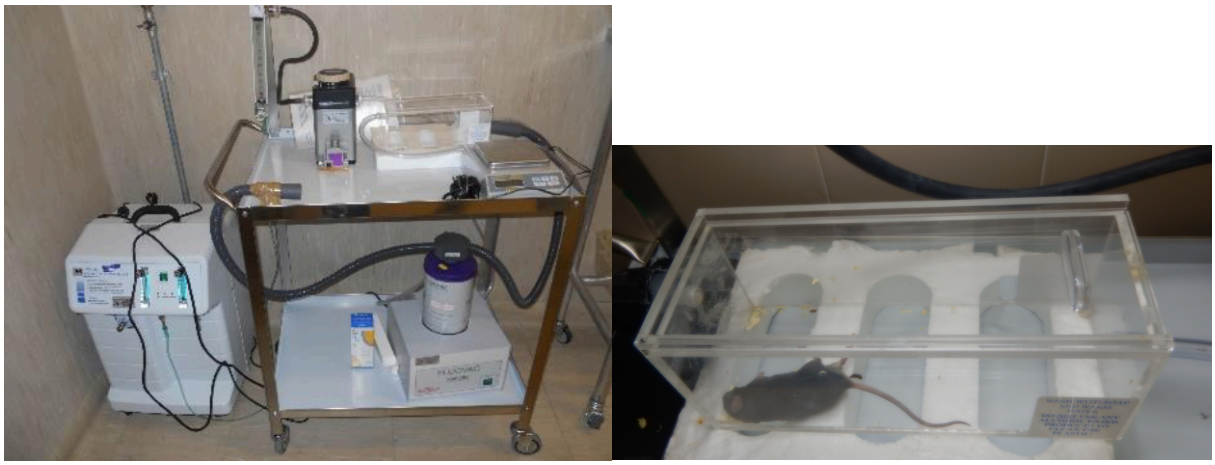


***Fig. 4.6**: **A)** Anaesthetic machine; **B)** Mouse in the induction chamber receiving 2.5% isoflurane in O_2.*

4.2.8 Humane Endpoints and Euthanasia

The humane endpoint is defined as "the point at which pain or distress in an experimental animal is prevented, terminated or relieved" (NRC, 2011). Animals should be euthanized at the earliest possible point that will provide experimental data in order to minimize suffering (Burkholder et al., 2012). Animals were euthanized when they reached a total score of 10 or if one of the signs was evaluated with the highest score, according to the humane endpoints (HE) table outcome score (**Table 4.3**).

Due to the effects of early senescence in homozygous and heterozygous animals, we expected to observe a number of clinical signs of natural aging that in another genotype at the same age would have indicated significant morbidity. In particular, considering that one of the aims of such research was the study of the lifespan, clinical signs of morbidity associated with aging were expected and necessary for the scientific aims and objectives of the study. Therefore, we tried to describe the humane endpoints as objectively as possible. However, when more subjective endpoints such as deterioration in general health or quality of life had to be used, the assessment relied on the veterinarian's observation and judgment in consultation with the operators to assess if the endpoint had been reached.

Euthanasia was carried out by administering an overdose of inhaled anesthetic (isoflurane 4%) in the induction chamber of the anesthetic machine.

Parameter	Animal Condition	Score
Feature	Normal	0
	Poor grooming, index of mild depression of the sensorium	1
	Matted fur	2
	Significant loss of fur, curved posture	3
	Lateral or abdominal decubitus or limb/limbs paralysis	4
Intake of food and water	Normal – Unknown: body weight < 5%	0-1
	Total anorexia: body weight < 15%	2
	Cachexia: poor general condition and evident weight loss	3
Respiratory symptoms	Normal respiratory rate	0
	Slight alterations of the respiratory rate	1
	Increased respiratory rate and abdominal breathing	2
	Decreased respiratory rate speed and abdominal breathing	3
	Marked abdominal breathing and cyanosis	4
Spontaneous behavior	Normal	0
	Slight alterations; excitability	1
	Decreased mobility and alert; solitary confinement	2
	Restless or very still; compulsive behaviours; circling, (repeated circular movements) as index of brain suffering	3
Induced behavior	Normal	0
	Mild sensorium depression or exaggerated response to stimuli	1
	Moderate changes in typical behavior	2
	Violent or extremely low reaction	3
Additional parameters	Rotated ears outwards and/or back; sharpened snout; narrow and half-closed eyes	4
	Rapid weight loss and severe dehydration	4
Total Score		

Table 4.3*: Standardized score table to evaluate animal suffering and define the humane endpoints. Experimental humane endpoints: the animal must be sacrificed when it reaches the score 10 of the table. The animal must be immediately sacrificed if it presents one of the signs evaluated with the highest score for a specific sign 4. The total score is classified as: 0-4 = Normal; 5-9 = Needs daily monitoring; 10 = Animal with initial distress signs; 11-13 = Animal with distress signs; ≥ 14 = Severe distress (National Research Council, Institute of Molecular Genetics, Bologna).*

4.2.9 Post-mortem Examinations

The animals died of natural cause or were euthanatized by means of painless method in compliance with the humane endpoints.

When possible, post-mortem examination was carried in the necropsy room of the Anatomo-Pathological Service of DIMEVET. However, when not possible necroscopy was conducted directly at the Laboratory Animal facility and the following organs were isolated and fixed in 10% neutral buffered formalin (NBF): lung, heart, thoracic aorta, kidney, liver, spleen, both interscapular and abdominal skin samples. All animals were subjected to histological examinations by the staff of the Anatomo-Patological Service of DIMEVET.

Skull and hind limb bones were analysed by the Institute of Molecular Genetics CNR and Rizzoli Orthopaedic Institute. The first was analysed by μCT, the latter by a mechanical study validated for the femur of such mouse model. Data on mechanical study of the femor are not shown because still very preliminary.

4.2.9.1 Histological Examinations

Wild type *Lmna*$^{+/+}$, heterozygous *Lmna*$^{G609G/+}$ and homozygous *Lmna*$^{G609G/G609G}$ mice were analysed. Tissues fixed in 10% NBF were dehydrated with graded alcohols and embedded in paraffin according to standard procedures.

Four-micron thick histologic sections were cut with a microtome and mounted on charged slides. Finally, the sections were stained with hematoxylin and eosin (H&E). Only on serial sections of aorta Periodic Acid-Schiff (PAS), and alcian blue (pH 1 and 2.5) stains were furthere planned to identify PAS positive glicoproteins or alcian blue positive at pH 1 sulphated mucins or alcian blue positive at pH 2.5 non-sulphated mucins. Observations were conducted using a microscope (Leica Microsytems SI, Cambridge CB1 3XJ, 12V/400MA), with magnification of 5x, 10x, 20x and 40x.

A specific grading system was used in order to depict skin and adnexa and aorta alterations (**Fig. 4.7 and 4.8**). In particular, skin grade 1 was considered normal with abundant adipose tissue in subcutis and numerous hair follicles having the hair bulbs in the adipose tissue layer; skin grade 2 had normal abundant adipose tissue but a mild reduction of hair follicles that start to lose their contact with subcutis; skin stage 3 had a complete atrophy or just scant multifocal remnants of adipose tissue and a moderate to severe reduction in hair follicles; skin grade 4 had a complete atrophy of adipose tissue and dermal fibrosis associated with rare hair

follicles (**Fig 4.7**). Aorta lesion were scored as grade 1 when showing normal feature with concentric multilayers (3-5) of leiomyocytes in the tunica media; grade 2 when showing presence of scant homogeneous and light eosinophilic material between the elastic fibers associated with multifocal decrease of cellularity in tunica media; stage 3 depicted a moderate multifocal discrete collection in tunica media interstitium of homogeneous and light eosinophilic material associated with a diffuse decrease in cellularity; grade 4 showed abundant eosinophilic material and severe decrease of cellularity (**Fig. 4.8**).

The organs were analysed by two blinded veterinary pathologists.

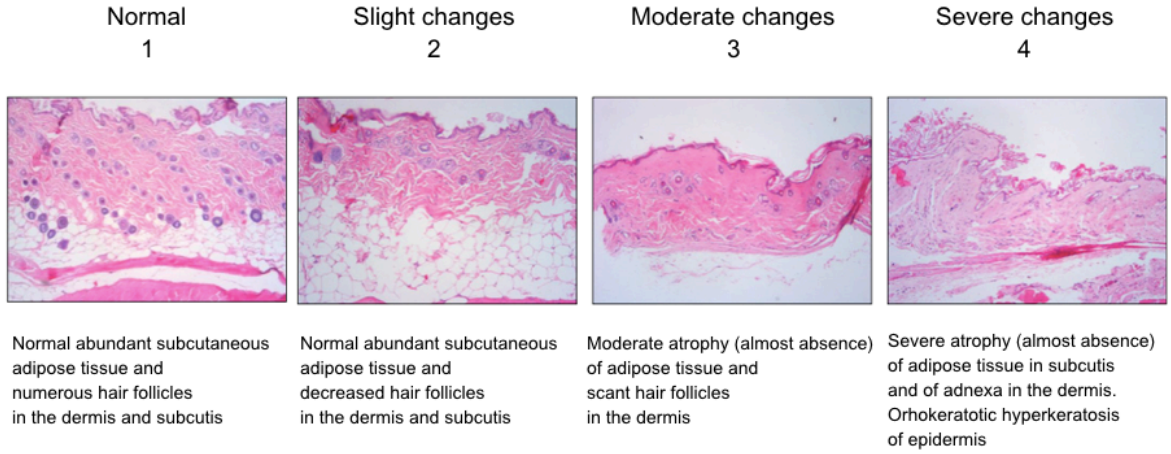


Fig. 4.7: *Grading of the skin and adnexa (10x).*

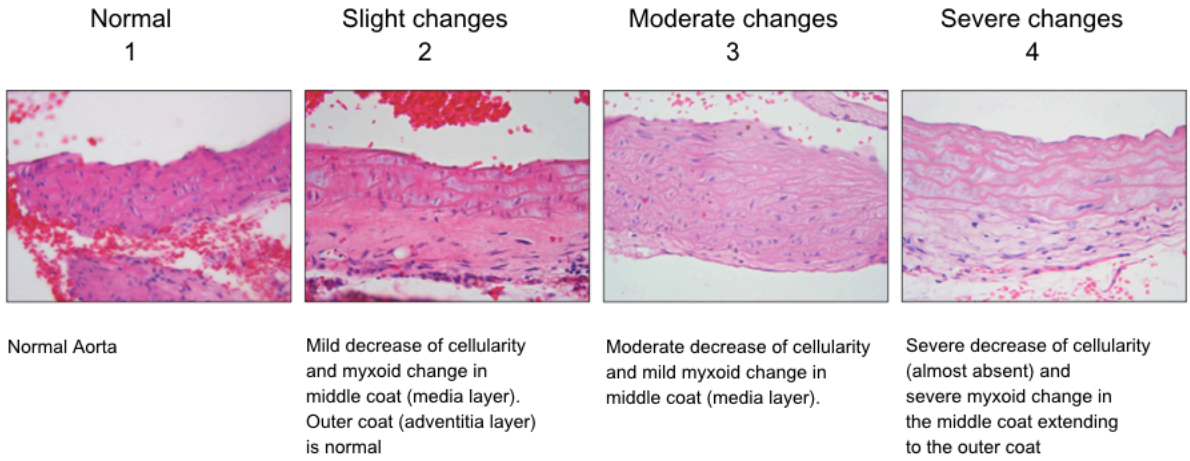


Fig. 4.8: *Grading of the myxoid lesion in the aorta wall (40x).*

4.2.9.2 Skeletal Analysis and Bone Matrix Evaluation

Skeletal morphological (µCT) examinations of the skull were performed at the Laboratory of Medical Technology Rizzoli Orthopaedic Institute (IOR). Such analyses were performed by a µCT Skyscan 1172 (mod. 1172, Bruker MicroCT, Konthich, Belgium) (**Fig. 4.9**). The instrument uses the cone beam method (Rüegsegger et al., 1996; Peyrin et al., 1998).

This method provides images from which three-dimensional models can be reconstructed, so it is possible to measure volumes, surfaces, thicknesses and distances.

Regarding the mouse skull, the samples were scanned in their test tube immersed in formalin. Each tube was wrapped in parafilm. The samples were scanned with 2 sub-scans, as they are larger than the field of view of the image.

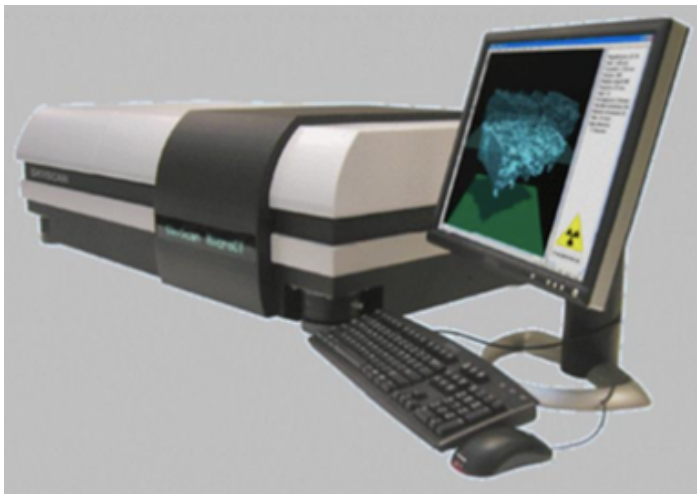


***Fig. 4.9**: µCT Skyscan 1172 (mod. 1172, Bruker MicroCT, Kontich, Belgium) for skeletal analysis. (http://www.directindustry.com).*

4.2.10 Statistical Analysis

Statistical analysis of the differences between mouse cohorts or different conditions was performed with a two-tailed Student's t-test. In experiments with more than two groups, differences were analysed by multifactorial one-way analysis of variance (ANOVA), and for the comparison of different groups in Kaplan-Meier survival plots, we used a log-rank (Mantel-Cox) test. Records of pup genotypes observed were compared with expectations from the Mendelian segregation of alleles using chi-square tests or a binomial test (two tailed). Microsoft Excel and GraphpadPrism 7 softwares for Macintosh were used for calculations and plots, and expressed the results as the means±SEM.

4.3 Results and Discussions

4.3.1 Nest and Breeding Characterization

The maintenance of the colony was guaranteed by 37 dams (7 *Lmna*$^{+/+}$ and 30 *Lmna*$^{G609G/+}$) and 25 males *Lmna*$^{G609G/+}$ over a 2-year period of study. The colony was maintained inbred and reached 8 generations. Our colony produced a total of 282 pups (**Fig. 4.10**), of which 15% were *Lmna*$^{G609G/G609G}$ (of these 6% females and 9% males), 51% *Lmna*$^{G609G/+}$ (of these 24% females and 27% males) and 32% *Lmna*$^{+/+}$ (equally distributed between females and males).

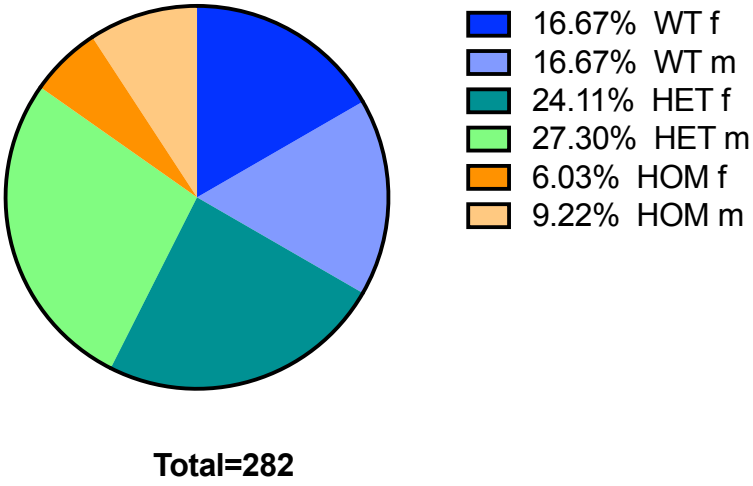


***Fig. 4.10**: Pie chart showing genotypes and sexes percentages of pups weaned in DIMVET's colony. WT = Lmna$^{+/+}$; HOM = Lmna$^{G609G/G609G}$; HET = Lmna$^{G609G/+}$; F = females; M = males.*

Weaned pups from heterozygous x heterozygous mating and heterozygous x WT mating are shown in **Fig. 4.11** and **Fig. 4.12**, respectively. As it can be seen, when heterozygous mice were mated together 32.5% of the weaned pups were wild type, 48% heterozygous and 19.5% homozygous. Chi-squared test revealed that the difference between observed versus expected genotypes was significant ($p = 0.0156$). Especially homozygous mice were less than what expected. When heterozygous were mated with wild type mice, weaned pups were 36% wild type and 64% heterozygous. Binomial test (two tailed) revealed that the difference between observed versus expected genotypes was significant ($p = 0.0479$). It is interesting to note that, in general, mice carrying the mutation were mostly males. In light of the above, it is not possible to establish, but it is fair to think, that in some cases especially homozygous and females carrying the mutation might have died in utero. It is interesting to note that also in humans the ratio females to males with HGPS is in favor of males, being 1:1.5 (DeBusk,

1972). In our colony, homozygous mice had a female to male ratio of 1:1.13, while heterozygous of 1:1.53, the latter being the same of the human ratio.

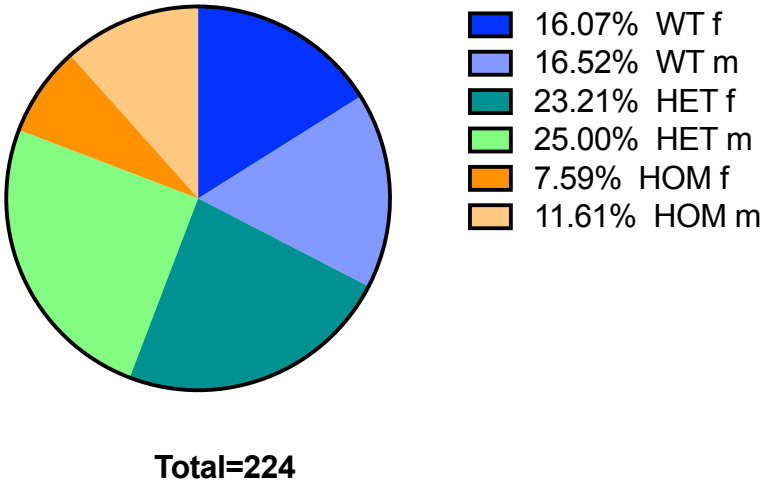


***Fig. 4.11**: Pie chart. Genotype and sex of pup weaned from Het mating. WT = Lmna$^{+/+}$; HOM = Lmna$^{G609G/G609G;}$ HET = Lmna$^{G609G/+}$; F = females; M = males.*

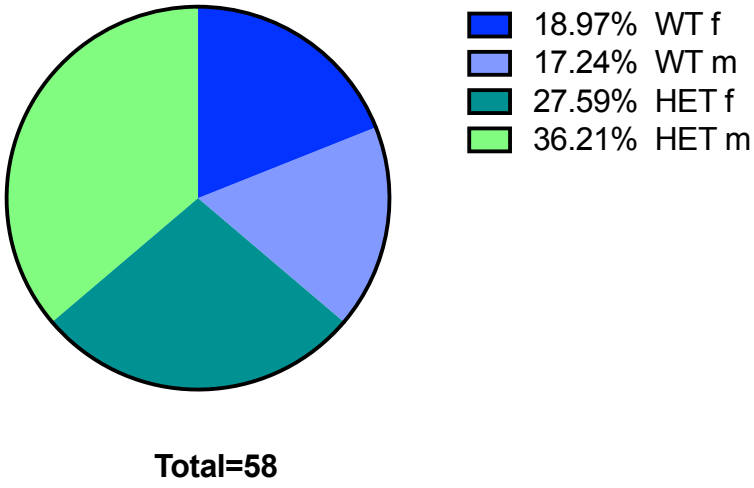


***Fig. 4.12**: Pie Chart. Genotype and sex of pup weaned from WT x HET mating. WT = Lmna$^{+/+}$; HOM = Lmna$^{G609G/G609G}$; HETt = Lmna$^{G609G/+}$; F = females; M = males.*

During their reproductive life, dams delivered the first successful litter starting from 57 days. Between births a mean of 36 days and 39 days was registered for *Lmna*$^{G609G/+}$ and *Lmna*$^{+/+}$ females, respectively. Considering that gestation period is usually of about 19 days for C57BL/6, the litters after the successful ones were probably lost, maybe due to the extended weaning period that made the reproduction cages often crowded and also to the fact that the dams did not have enough milk to feed all pups.

Our hypothesis that *Lmna*$^{+/+}$ would be better damns compared to *Lmna*$^{G609G/+}$ was not confirmed. In fact, both genotypes had an average of 2 litters during their reproductive life (range 1-4). Litter size were highly variable, ranging from 1 to 11 pups, however, for both genotypes, the average was 6 pups for each litter, which is more than what reported for C57BL/6J (Jackson Laboratory, 2007). Therefore, heterozygous males and females revealed to be reliable breeders. On the other hand, one of the two homozygous females in reproduction was never pregnant during a three-month period, while the second one got pregnant but died during premature delivery, together with fetuses. The homozygous male in reproduction did not succeed in impregnating two fertile heterozygous females. In light of this result and since generally mice reach sexual maturity at about 60 days of age, which is an already senescent age for homozygous *Lmna*$^{G609G/G609G}$ transgenic mice, they cannot be considered as breeders. However, they are not necessarily infertile as stated by Osorio et al. (2011).

The total pre-weaning pup mortality was around 31%. This pre-weaning pup mortality is in accordance with previous studies conducted on C57BL/6 mice (Weber et al., 2013), even though mortality rates vary greatly among different studies (Weber et al., 2016). Considering that mouse pups are born without fur and therefore they are very sensitive to hypothermia (Weber et al., 2016), and that animal models of laminopathies have been proven to benefit from higher temperatures (Liao et al., 2016), we assumed that providing appropriate nesting material is very important to reduce pup mortality in our colony since maternal behavior, including nest building attitude, was not impaired. However, some pup deaths in the first few days after birth likely went unnoticed, since dams often scavenge dead pups. Furthermore, we tried to leave periparturient females undisturbed, which may have delayed or prevent the discovery of pup loss. In some cases, as previously said, rejection or cannibalism of the litter were observed, probably due to a lack of breast milk evidenced by the absence of “milk spot” in the dead pups. Such deaths commonly occurred by 1 or 2 days of age.

Since birth, transgenic pups for the mutation evidenced a slower growth in comparison to *Lmna*$^{+/+}$, suggesting lengthening the weaning period to ~30 days. Although, Baumans (2007) suggests weaning at 21 days, C57BL/6J mice are reported to normally benefit from being weaned at 28 days (Jackson Laboratory, 2007). Our strategy to wean at around 30 days of age resulted successful, and no post-weaning mortality was registered.

The headset marking revealed to be a fast and easy method to identify animals. Furthermore, no ear dermatitis, often related to ear tags used for identification (Burkholder et al., 2012), was registered.

In cages overpopulated with pups from different litters, barbering of suckling pups by their mother or father was common (**Fig. 4.13**). After weaning, the fur of pups with barbering grew normally. No barbering was observed for breeding pairs, as also for "maternal" barbering in lactating mice performed by suckling pups.

***Fig. 4.13**: Barbering of suckling pups by their parents.*

4.3.2 Colony Health Surveillance

The two sentinels tested were negative to most of the relevant pathogens tested. However, they revealed that the colony was positive for *Helicobacter hepaticus*, *Helicobacter rodentium*, *Helicobacter typhlonius* (all detected through PCR), and *Tritrichomonas* spp. (detected through microscopy), detected in the intestinal material through PCR and microscopy analysis, respectively. Naturally acquired *Helicobacter* infections have been reported in all commonly used laboratory rodent species (Chichlowski & Hale, 2009; Charles River, 2017a). A study of mice derived from 34 commercial and academic institutions in Canada, Europe, Asia, Australia, and the United States showed that 88% of these institutions had mouse colonies infected with 1 or more *Helicobacter* spp. (Taylor et al., 2007). *Helicobacter* infections could potentially interfere with *in vivo* experiments and biomedical research by affecting mainly the gastrointestinal system, in minor entity the reproductive system, and by developing some types of cancers (Chichlowski & Hale, 2009). However, no clinical symptoms, necroscopic signs of intestinal inflammation nor cancers were found in our colony that could have been linked to *Helicobacter* spp. infections, meaning that it is unlikely that these infections could have

interfered with the results of the study. *Tritrichomonas* spp. are common protozoa in the intestinal tract of rodents and are not considered to be pathogenic (Charles Rivers, 2017b). Once again, no clinical signs associated to protozoal infections were detected.

In light of the infections detected, we decided to not treat the animals since we evaluated that the risk/benefit ratio of treating was in risk's favor. Furthermore, the treatment could have interfered with the treatment study described in the next chapter. The animals, despite the signs linked to the genetic disease, always resulted clinically healthy and no necroscopic lesion could be linked to the above-mentioned infections.

One of the two sentinels screened also resulted positive to *Pasteurella pneumotropica* infection, isolated through culture. Such gram-negative coccobacillus is quite common in laboratory populations, and usually infections are asymptomatic (Charles Rivers, 2017c). In our study, the only sign that could have been liked to this infection is the conjunctivitis seen in some animals. However, if clinically healthy, animals infected with *Pasteurella pneumotropica* are considered suitable for research purposes (Charles Rivers, 2017c).

It is important to have knowledge of the colony infection status in order to eventually link the results to the infections and not the disease studied.

It is interesting to note that ulcerative dermatitis (UD), an idiopathic, spontaneous, debilitating syndrome of laboratory mice, is typically a disease of aged C57BL/6 mice or genetically engineered mice on a C57BL/6 background, with prevalence rates ranging between 4.1% to 21% (Hampton et al., 2012). However, genetic factors in sub-strains of C57BL/6 mice may play a role since very different results for prevalence, age and sex onset of UD were reported in different studies (Sundberg et al., 2011; Hampton et al., 2012). During our study, no lesions ascribable to ulcerative dermatitis were evidenced.

4.3.3 Behavior and Clinical Signs

C57BL/6 strain is usually "touchy" (Baumans, 2007), however in our *Lmna*^{G609G} colony no aggressive behavior was ever seen towards the operators and mice acted comfortable when manipulated. Few aggressive behaviors were observed between cage mates. These were sporadic exceptions, observed only in males that fought to form a hierarchy especially after litter change. This behavior was limited by adding some old litter or paper tissue from the old cage into the new one. The week territorial behavior made it possible to cage males in groups up to 4 animals. In those rare cases in which animals were found with fight wounds (**Fig. 4.14**),

the animal responsible was identified and isolated from others. Animals with wounds were properly disinfected with iodate solution and wounds quickly healed without complications.

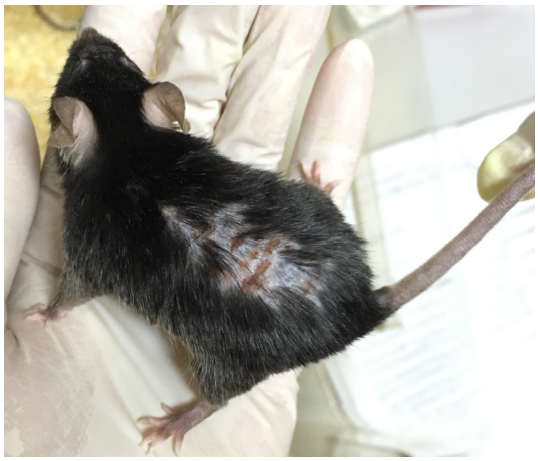


***Fig. 4.14**: Fight wounds.*

Behavior-associated hair loss, known as barbering, includes plucking of fur or whiskers from cage mates (hetero-barbering) or onself (self-barbering), and is common in mice (Kalueff et al., 2006). In particular, it occurs often in C57BL/6 suggesting a strong genetic component (McElwee et al., 1999; Kalueff et al., 2006). In general, it is thought to be linked to a form of dominance in which animals co-operate (van den Broek et al., 1993). Barbering has also been negatively correlated with signs of aggressiveness (Kalueff et al., 2010) and is interpreted as a way to maintain social hierarchy in colonies. In other studies, it has been interpreted as an obsessive compulsive grooming disorder, often representing a stress-evoked response that can be limited with environmental enrichment. In our colony, adult group-housed mice displayed barbering in 12% of the cases (**Fig. 4.15**), which is lower that what reported for C57BL/6 in other studies (Long, 1972). Barbering behavior was not related to a specific genotype. Most frequent location of hair loss observed throughout the study was on the snout, neck, and head, which cannot be explained by self-barbering. Since all animals with barbering had healthy skin, no ulceration or inflammation no medical treatment was necessary. Our colony might have minimized potential aggression with this social behavior since fight wounds were found in very rare cases. However, in some occasion increasing environmental enrichment diminished the barbering confirming that this behavior in multifactorial and that it can be stress-evoked.

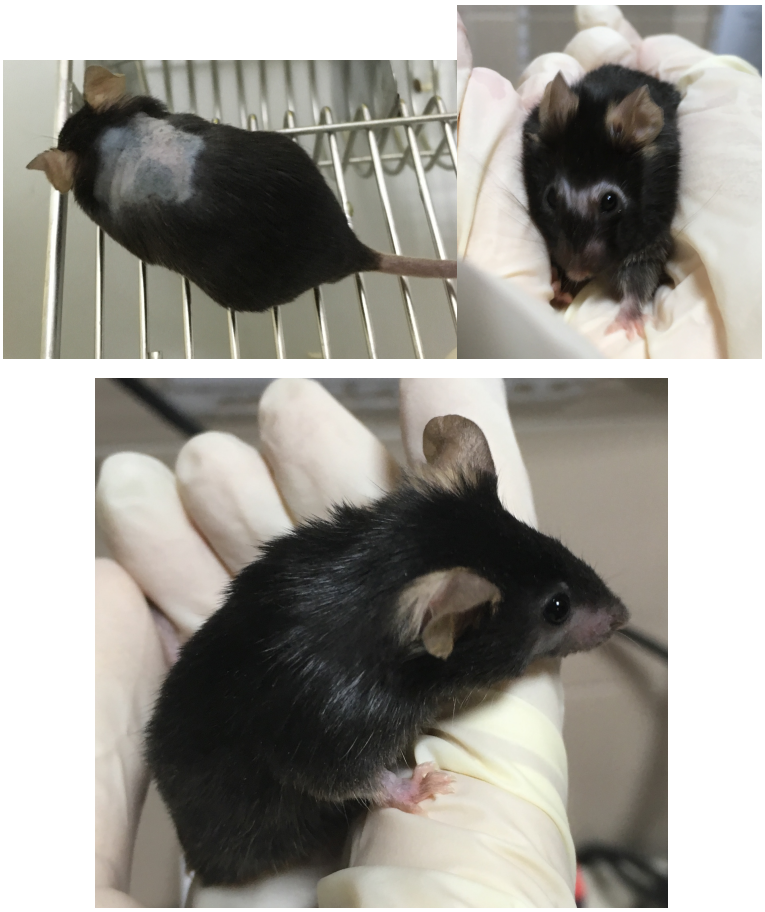


***Fig. 4.15**: Barbering. Note that the skin is healthy in these cases. Upper left: bald patch on neck and upper back. Upper right: barbering around the eyes and whiskers removal. Below: snout and whiskers barbering. Front leg self-barbering.*

Following weaning and for the first 5-6 weeks of age, all mice were lively, active, explorative and expressed the typical behavior of the species. When observed in their home cage mice were seen moving around the cage, grooming, eating, drinking and interacting with cage mates. All mice built nests using the suitable material provided, such as sizzle nest, tissue, and egg packs. They also could be seen enjoying playing with the plastic or cardboard rolls provided (**Fig. 4.16**).

***Fig. 4.16**: Housing enrichment. Cardboard and plastic rolls, egg packs, paper tissue.*

Apart from weight loss (as reported below), the first clinical signs associated with progeric syndrome arose at 5-6 weeks of age for *Lmna*$^{G609G/G609G}$ and at 20-32 weeks of age for *Lmna*$^{G609G/+}$. These signs were noted during daily observation of the animals in their home cage and during the general body examination conducted twice weekly. The signs regarded especially the fur and the skin, the eyes and the skeletal system.

For both homozygous and heterozygous mice, periocular alopecia was the first characteristic sign registered, followed by thinning and loss of hair from the limbs, nose and back (**Fig. 4.17**). Hair fragility and loss were evidenced by its presence on the gloves when animals were handled. Fur was evidently opaque, scruffy and rough compared to *Lmna*$^{+/+}$ and with premature grey/white strikes (**Fig. 4.17**).

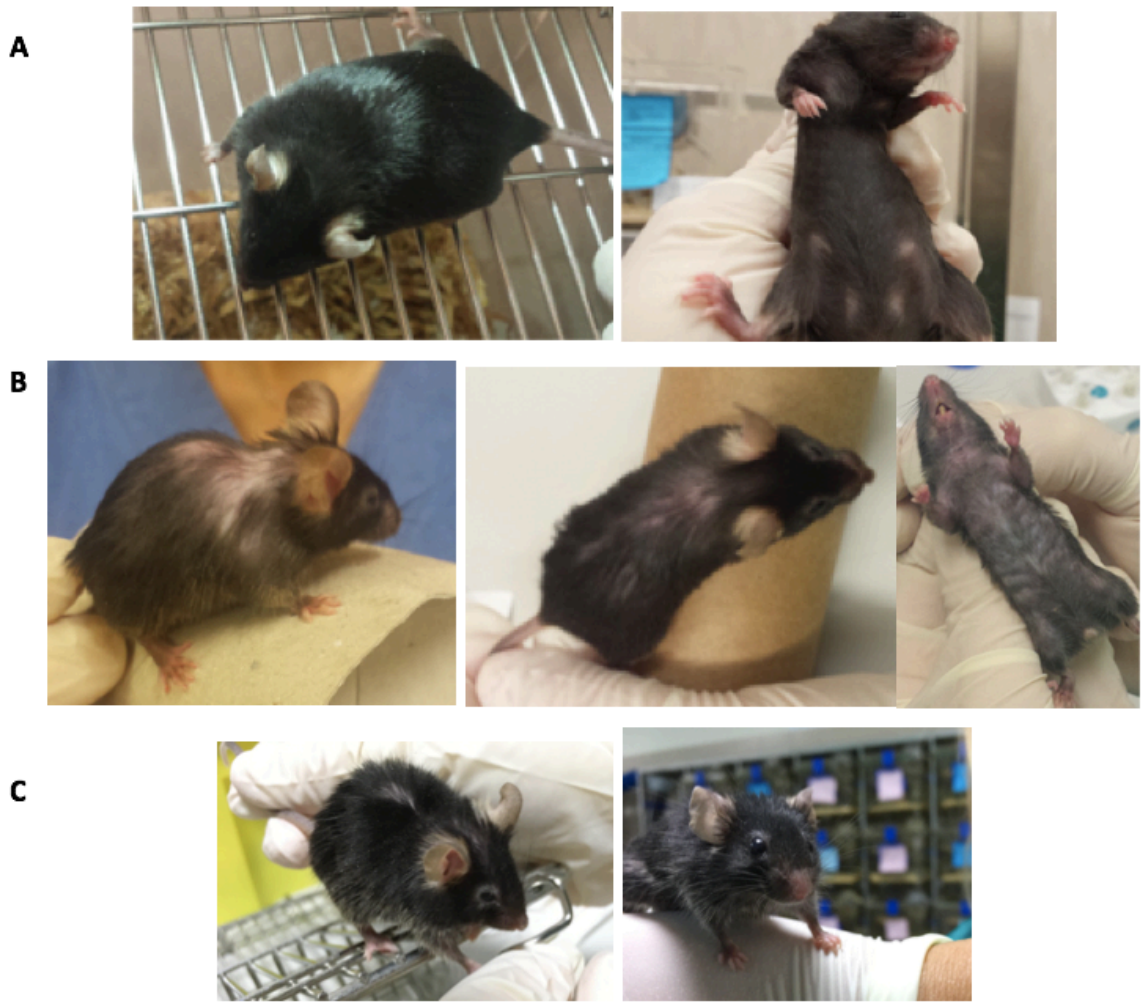


***Fig. 4.17**: Fur comparison between Lmna$^{+/+}$ mice **(A)** and Lmna^{G609G} transgenic mice **(B, C)**. Note the thick, shiny and glossy fur of the Lmna$^{+/+}$ mice both in the dorsal and ventral area **(A)**. In contrast, G609G transgenic mice have sparse and scruffy coat. Trunkal hair turned prematurely grey **(B)**. Note the characteristic periocular alopecia **(C)**.*

The skin appeared to be thin, flaky, dry and sclerotic, and an erythema-like redness was clearly visible, particularly in the ventral area (**Fig. 4.18**). The irritation in the ventral area could have been enhanced from the contact of the litter with the skin considering the sparse fur on it. The severeness of the phenotype was much more evident in homozygous mice than in heterozygous.

Although rarely, dystrophic nails were observed (**Fig. 4.19**).

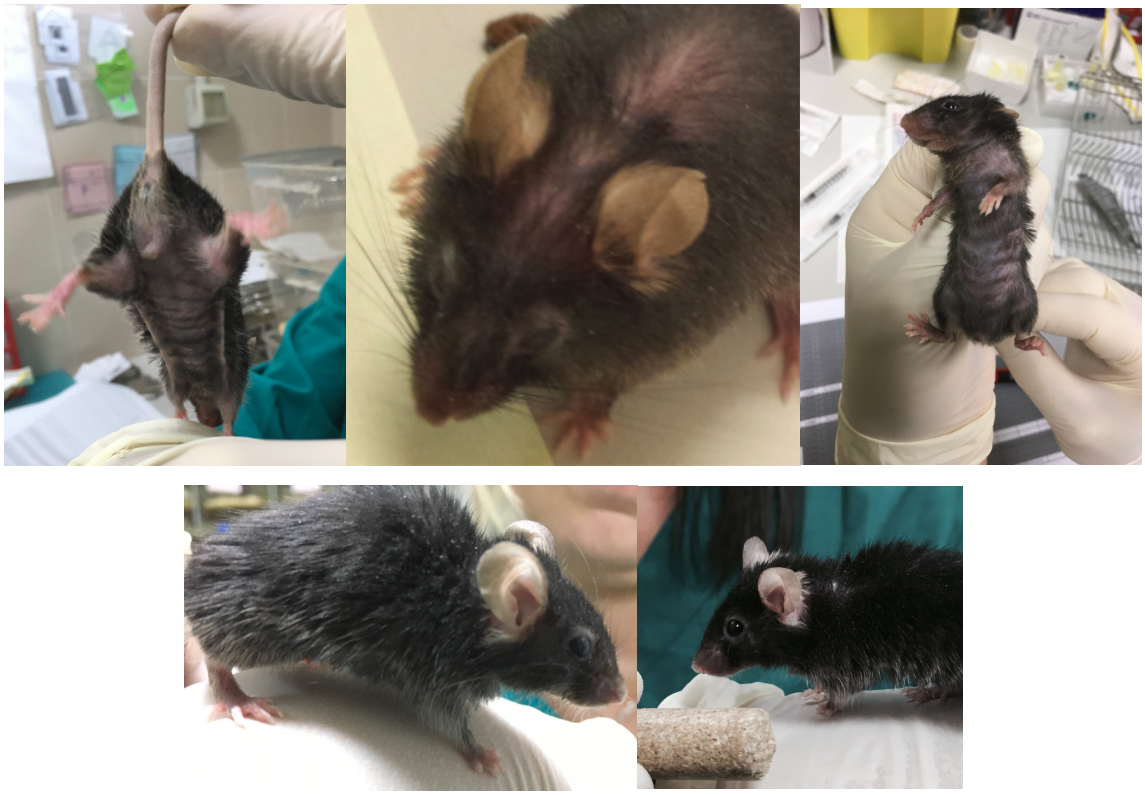


***Fig. 4.18**: Skin and fur in Lmna^{G609G} transgenic mice. Above: Thin fur and erythema-like redness. Below: Opaque and dry fur. Streaked with grey, especially in the ventral area.*

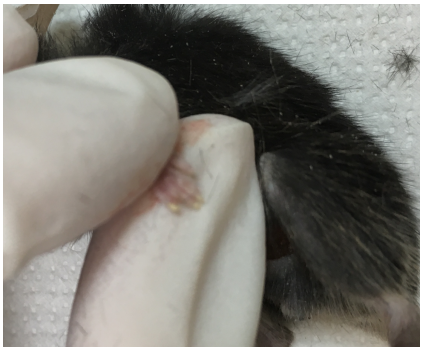


***Fig. 4.19**: Dystrophic nails in a heterozygous mouse.*

Ocular signs, never described in human patients, were very common. These signs included microphthalmia, anophtalmia, opacity of the cornea, and occurred in 50% of homozygous mice (**Fig. 4.20**). However, they were also evidenced in the heterozygous (17%), and more rarely in wild type (3%). C57BL/6 are known to have high incidence of microphthalmia and other eye defects (Smith et al., 1994; Fuerst et al., 2007; Burkholder et al., 2012), making it difficult to attribute these observation to the *Lmna*^{G609G} transgenic mice and possibly being the expression of the genetic background.

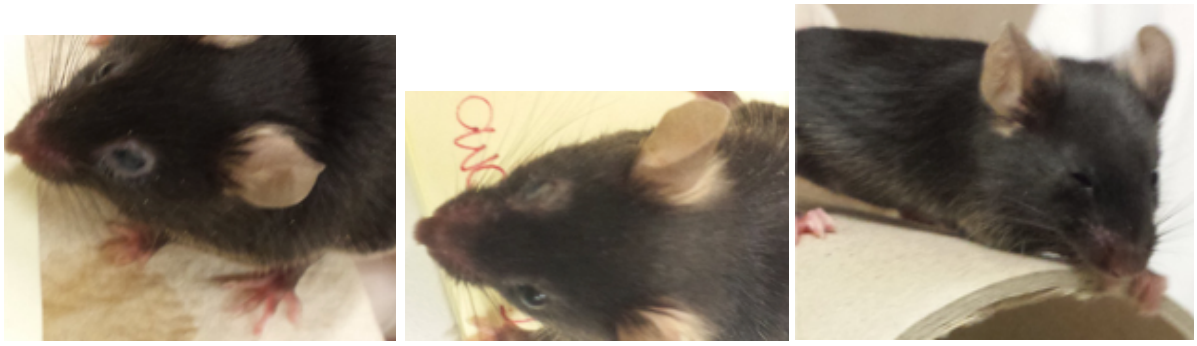


Fig. 4.20*: Ocular signs in Lmna*^{G609G} *transgenic mice. Note periocular alopecia* ***(A)****, opacity of the cornea* ***(B)****, microphtalmia* ***(C)****.*

Transgenic mice also showed dental anomalies, confirmed by radiological and µCT analysis (**see Paragraphs 4.3.7 and 4.3.9, Fig. 4.41 and 4.5**). Mouse teeth's grow throughout life and should meet in such a way that they grind on each other and on the feed to remain at a normal length. If the mandibular and maxillary teeth are not normally aligned and do not properly cloose, malocclusion occurs meaning that teeth may grow into the palate or out of the mouth. Malocclusion has been linked to trauma to developing teeth (cage lids, improper handling, fighting, too-hard food), but also a genetic basis is suggested by an increased incidence in certain strains, such as C57BL/6 (Fox et al., 2007b; Burkholder et al., 2012). In our study, we never found malocclusion, nor other dental alterations, in *Lmna*$^{+/+}$ mice, while the incisors were splayed apart, thin, long or fractured in 13% of homozygous and 8% of heterozygous (**Fig. 4.21**) This condition was early diagnosed during the twice weekly clinical observation and malnourishment was an obvious consequence of malocclusion. In some cases, incisors grew splayed apart, thin and were prone to fractures (**Fig. 4.21**). Cleft palate was not observed.

***Fig. 4.21**: Normal teeth **(A)** compared to dental anomalies. **B)** Malocclusion, upper incisors extremely angled inwards; **C)** Long incisors splayed apart; **D)** thin, long and fractured incisors; **E)** fractured lower incisors and long upper incisors.*

Kyphosis (**Fig. 4.22**), reduced mobility and a "shuffling gait" of the hind limbs were assessed by clinical observation. Kyphosis was also assessed by X-rays (**see Paragraph 4.3.7**).

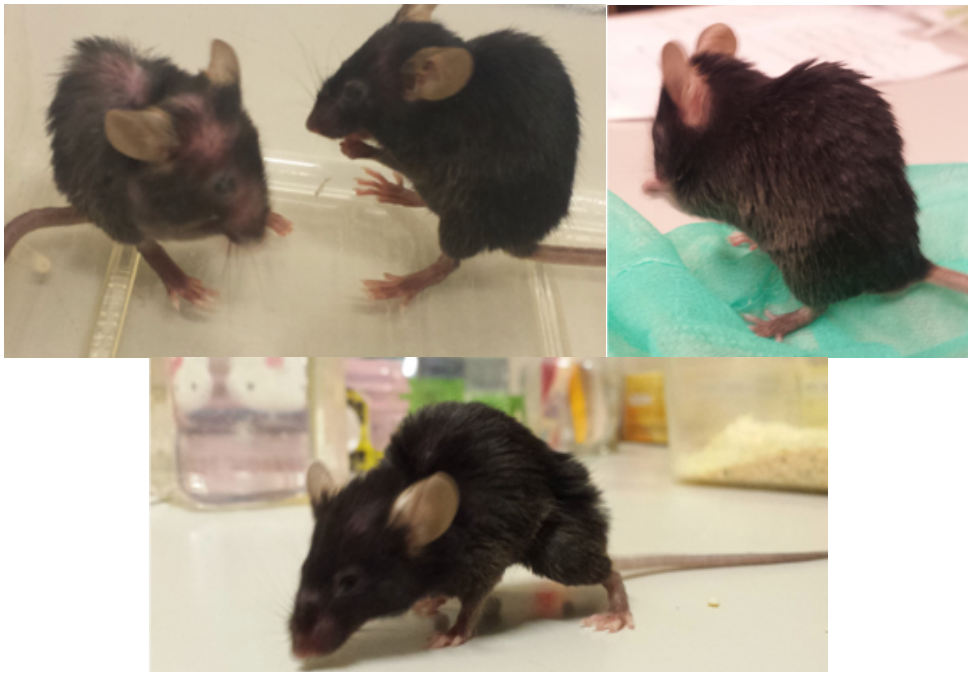


***Fig. 4.22**: Kyphosis in Lmna^{G609G} transgenic mice.*

C57BL/6J mice tend to have more pups with hydrocephalus compared to other strains (Jackson Laboratory, 2007). This is a condition in which fluid builds up in the ventricles of the brain and does not distribute normally between the brain and the spinal cord (Burkholder et al., 2012). In our colony, 2 heterozygous mice were excluded from the study and euthanized before adulthood because were born with hydrocephalus (**Fig. 4.23**). Visibly these mice had a large rounded head and shortened muzzle since weaning. They were smaller than littermates and with time they developed lethargy and neurological abnormalities. During the OFT (data not presented) they would never stay still or do vertical movements, grooming or freezing, and would continue running compulsively in the peripheral area attached to the side of the arena, going only in one direction.

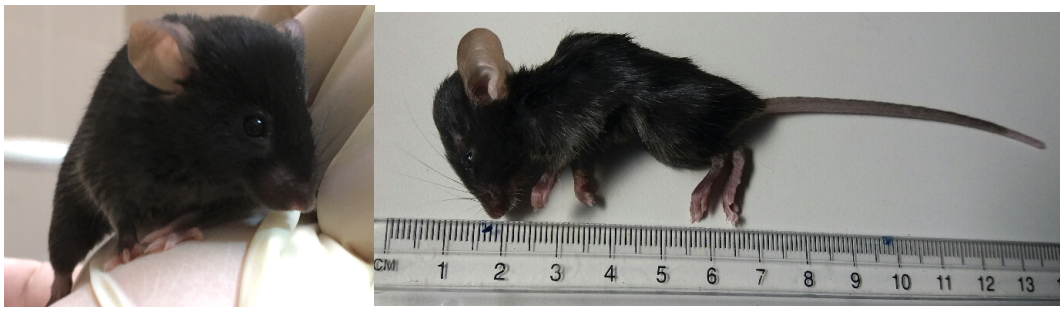


***Fig. 4.23**: Hydrocephalus in a Lmna$^{G609G/+}$. Left: 1.5 month old; Right: 2.5 month old.*

Dental anomalies, together with the reduced motility, reduced agility in climbing and/or standing to reach food, resulted in feeding difficulty for the affected animals. Consequent weakness and weight loss led to a severe state of apathy and cachexia rapidly reaching the HE. Starting from the end of March 2016 until July 2016, for a subset of mice (G1-G30) moistened chow was left on the cage floor, a more accessible position, in order to avoid feeding difficulties (**Fig. 4.24**). The strategy of adding palatable food on the cage floor is used by many research groups and is part of the refinement principle (Burkholder et al., 2012). In our study, this choice brought to good results because a general increase in body weight was observed, allowing the less motile animals to continue to eat (**Table 4.4**). This procedure was therefore considered to be useful to improve life conditions of the affected animals. However, adding moistened chow

***Fig. 4.24**: Moistened chow. Homozygous mice eating.*

was useful only for about two weeks after the initial weight loss. In fact, various factors linked to the disease eventually compromised the animal wellness and nutrition. Moistened food did not have an impact on lifespan (**see Paragraph 4.3.5**). Since this technique improves life quality of mice without interfering with lifespan it is considered a valuable refinement strategy to use in future studies.

Animal/Date	18/3	21/3	23/3	25/3	30/3	1/4	6/4	8/4	12/4
L1G6 M Het	27,5	21	21,1	21,8	19,9	23,7	23,9	25,2	25,8
L3G6 M Het	28,2	28,1	28,2	28,3	27,1	27	27,7	29,7	28,8
L4*5*G6 M Het	25,9	25,3	25,1	24,6	24,9	25,1	25,8	27,1	26,5
L1G14 M Het	28,7	29,3	30,1	28,3	29,4	29,1	29,1	31,8	31,4
L2G14 M Het	33,4	33	32,1	33	33,4	32,9	35,4	35,5	35,8
L3G16 M Homo	20,2	19	16,5	18,9	18,8	18,1	18	20,6	20,8
L1G17 F Homo	15,2	15,5	15,9	16,1	14,8	18,6	18,1	18,5	18,8
L2G17 F Het	16,8	17,1	17,8	18	13,9	19,9	19,9	19,9	19,9
L3G17 F Het	15,4	16,5	17,2	17,9	14,6	19,2	19,6	20	20,4
L1G20 M Homo	16,1	16,8	17	18,2	18,7	18,9	18,2	20	17,3

***Table 4.4**: Body weight changes of some heterozygous/homozygous mice before and after taking wet feed (March-April, 2016). The boxes highlighted in green show the increase in body weight.*

Tremors - involuntary rhythmic oscillation of body parts - were observed in 2/40 homozygous mice and 2/157 heterozygous mice. They were never observed in wild type mice. Tremors could be related to a possible hypoglycemic state or could derive from a neurological disorder (Louis, 2008). Moderate to severe hypoglycemia, not investigated in this study, was described for homozygotes and heterozygotes at 3 and 8 months of age, respectively (Osorio et al., 2011). However, it is possible that shivering may have been confused for tremors. Since homozygotes and heterozygotes have thin fur and no fat the shivering could be a physiological response to hypothermia. Liao et al. (2016) demonstrated that termoneutrality (30°C) improved lifespan of a mouse model of laminopathies, reinforcing the idea that these mice could be more severely influenced by the temperatures commonly used in laboratory animal facilities. The fact that animal models of laminopathies benefit from higher environmental temperatures was also communicated during the International Meeting on Laminopathies 2017 (oral communication) by different research groups working with such models.

Overall, clinical signs related to the mutation were evident in both phenotypes. The fairly milder phenotype of *Lmna*$^{G609G/+}$ mice, compared to *Lmna*$^{G608G/+}$ HGPS patients, is in agreement with previous observations evidencing a higher tolerance of mice compared to humans to accumulation of prelamin A forms (Osorio et al., 2011). Both genotypes are reliable models of HGPS.

4.3.4 Body Weight and Growth

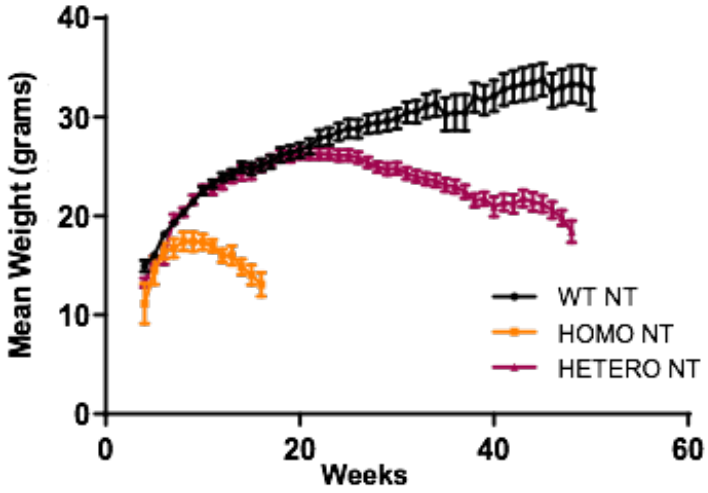


***Fig. 4.25**: Cumulative plot of body weight versus age. Dots represent mean values, and bars indicate SEM. N=67 wild type mice, n=10 homozygous mice, and n=52 heterozygous mice.*

As it can be seen in **Fig. 4.25** HGPS wild type colony grew rapidly since birth and after weaning, reaching a relative plateau weight of 35 g for males and 25 g for females at 25 weeks. At 8 weeks of age females weighted already 4.1±0.6 g less than males making weight sexual dimorphism quite evident. All wild type mice did not show any weight loss during the study period and were able to reach up to 50 g.

As noted before (Osorio et al., 2011), HGPS colony transgenic for the mutation attained lower weights than respective wild type mice throughout the post-weaning period. In particular, between 45 and 48 days of age, mean values of each group indicated lower weights for both females and males *Lmna*$^{G609G/+}$ compared to wild type of the same sex, in particular 0.6±0.4 g less for females and 0.6±0.6 g less for males. It is intuitive that this slight weight difference was hard to detect at weaning. The weight difference was more evident for *Lmna*$^{G609G/G609G}$, males weighting 3.6±0.7 g less than *Lmna*$^{+/+}$ males, and females weighting 2.3±0.4 g less than *Lmna*$^{+/+}$ females, registered always between 45 and 48 days of age. The first sign associated with HGPS disease, in fact, included reduced growth rates.

Since the number of animals enrolled in this phenotypic study were more than in previous studies, and since the sexual dimorphism was so evident and could interfere with the evaluation of data if considered cumulatively, we analyzed females and males in separates plots of body weight versus age (**Fig. 4.26**).

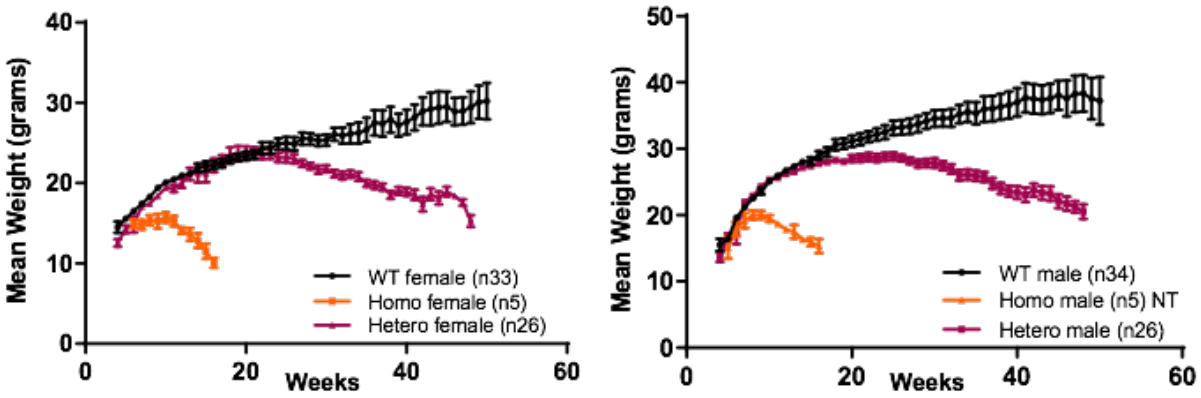


***Fig. 4.26**: Cumulative plot of body weight versus age in females (left) and males (right). Dots represent mean values, and bars indicate SEM.*

As it can be seen from **Fig. 4.27**, within the same genotype sex differences in weight were always significant ($p < 0.05$).

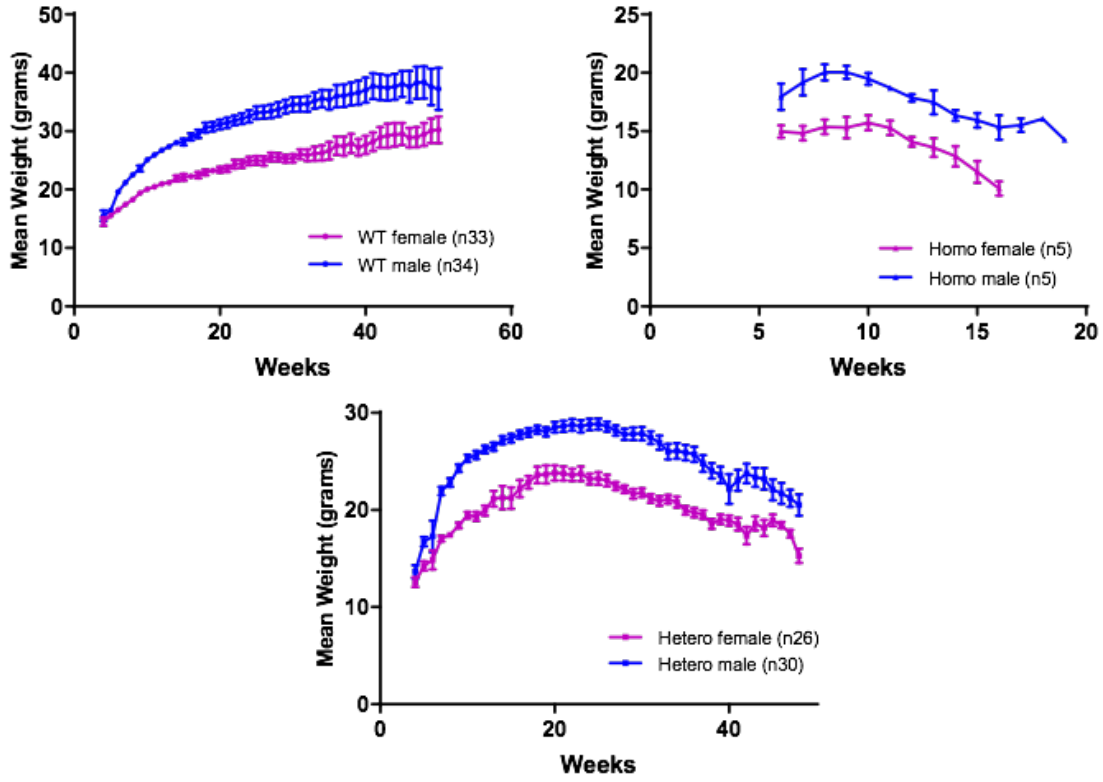


***Fig. 4.27**: Cumulative plots of body weight versus age in the three genotypes (upper left: wild type; upper right: homozygotes; below: heterozygotes), distinguished by sex. Dots represent mean values, and bars indicate SEM.*

In accordance with the first description of these animals, no weight plateau was reached for homozygous. Males and females hardly reached a maximum of 20 g and 15 g, respectively, at about 9 weeks of age which was followed by a rapid weight loss. Contrarily, heterozygous mice

reached a plateau (males *Lmna*$^{G609G/+}$ 28 g; females *Lmna*$^{G609G/+}$ 23 g) at about 20 weeks and maintained this weight until 30 weeks of age, when they progressively started to lose weight. Differences in sizes, and in weights between the *Lmna*$^{+/+}$, *Lmna*$^{G609G/+}$ and *Lmna*$^{G609G/G609G}$ were quite evident, as it can be seen in **Fig. 4.28**.

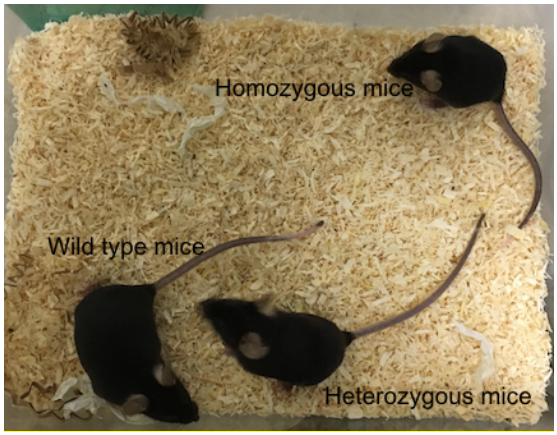


***Fig. 4.28**: Size comparison between Lmna$^{+/+}$, Lmna$^{G609G/+}$ and Lmna$^{G609G/G609G}$ females from the same litter at 2 months of age.*

4.3.5 Lifespan

The lifespan of *Lmna*^{G609G} transgenic mice is very short compared to a *Lmna*$^{+/+}$ (**Fig.4.29**). C57BL/6 mice usually survive 18-22 months, females living longer than males (Russel, 1966). However, longevity is strain specific, and depends on several other factors resulting quite variable among research groups. The oldest *Lmna*$^{+/+}$ mice in our study was a female and lived up to 23 months.

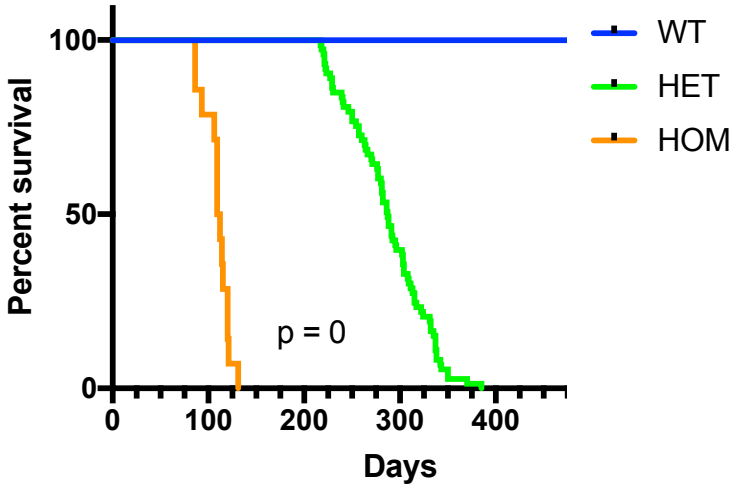


***Fig. 4.29**: Kaplan-Meier survival plots for wild type mice (blue, n= 15), heterozygous mice (green, n=73), and homozygous mice (orange, n=15).*

Homozygous mice (n=15) with the c.1827C>T;p.Gly609Gly mutation presented a premature death, reaching the humane endpoint at 108±4 days (**Fig. 4.29**). This result is consistent whith those reported by previous studies, in which mean lifespan ranges from 103 days to 107 days (Osorio et al., 2011; Osorio et al., 2012; Villa-Bellosta et al., 2013). If considered separately, females (n=8) had a lifespan of 101±5 days, while males (n=7) had a lifespan of 115±5 days. This difference between the two sexes of the same genotype resulted significant (p = 0.0131) (**Fig. 4.30**). Although moistened chow helped reducing weight loss (**see Paragraph 4.3.3**), it did not affect homozygous mice lifespan in a significant way (p = 0.46), meaning that this can be considered a good refinement technique not necessarily influencing life expectancy during drug testing.

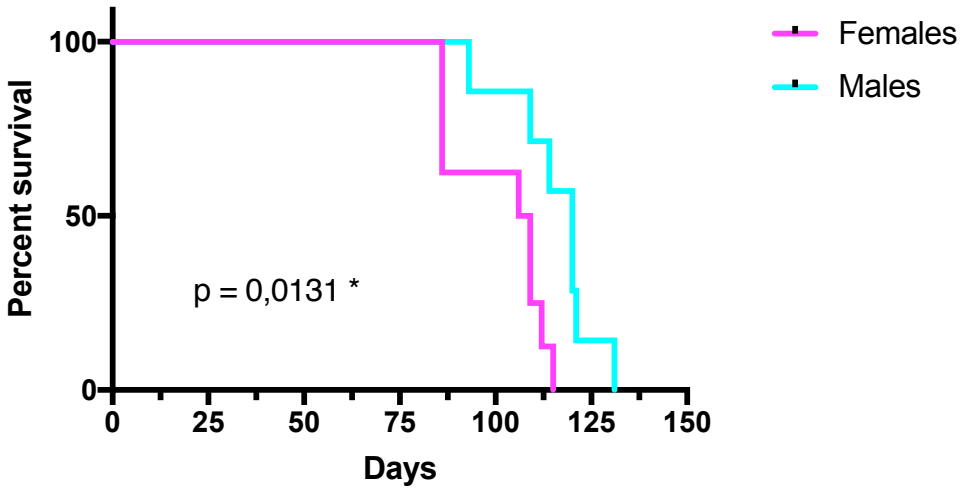


***Fig. 4.30**: Kaplan-Meier survival plots for homozygous females (pink, n=8), and males (light blue, n=7).*

Heterozygous mice (n=73), which develop the disease in a less severe way, reached the humane endpoint at 287±5 days (**Fig. 4.29**). Osorio et al. (2011) and Villa-Bellosta et al. (2013) reported a mean lifespan of 242 and 238 days, respectively. Once again, in our study females reached the humane endpoint earlier compared to males (**Fig. 4.31**). In fact, females (n=40) reached the HE at 266±6 days, while males (n=33) at 311±6 days (p < 0.001). Finally, also for heterozygous mice, moistened chow did not prolong life expectancy in a significant way (p = 0.5).

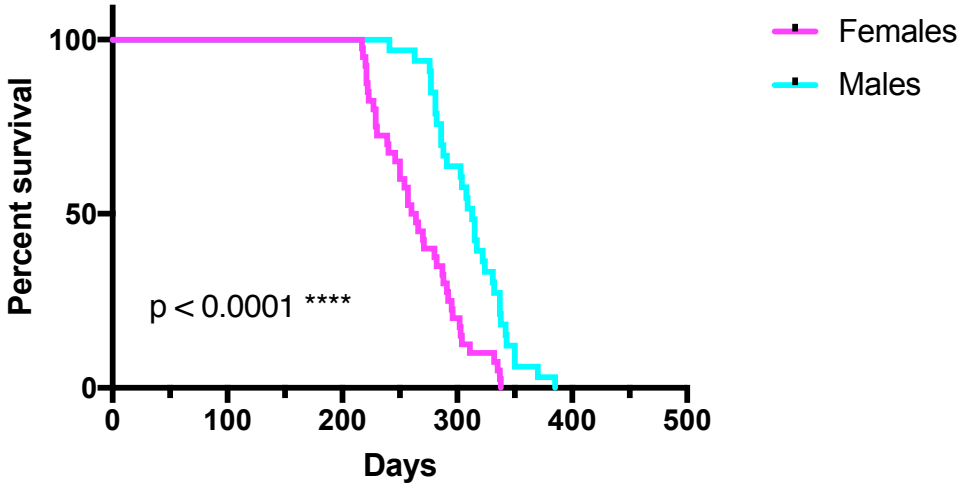


***Fig.4.31**: Kaplan-Meier survival plots for heterozygous females (pink, n=40), and males (light blue, n=33).*

Considering dams and virgins separately, we noted that while virgins (n=30) lived 272±6 days, dams (n=10) lived 250±10 days. Shortened lifespan for breeding females has already been reported (Russel, 1966). On the other hand, means of male lifespan were not affected at all by reproduction. However, for both sexes, breeding did not affect lifespan in a significant way. In general, it has to be noted that in our study mean lifespan of *Lmna*$^{G609G/+}$ was longer than in previous studies. In such studies (Osorio et al., 2011; Villa-Bellosta et al., 2013), no range±SEM for lifespan was reported and groups were small in numbers, having not more than 8 animals each. From their survival plots it can be deduced that ranges were quite wide. For this reason, we think that our results could be more reliable for individuating the real mean lifespan of *Lmna*$^{G609G/+}$. Differences in mean lifespan of mice from the same inbred strains in different environments, demonstrate that life expectancy is influenced not only by genetic factors, but also by the environment. These factors, among others, include diet, temperature and humidity conditions, and husbandry procedures. Obviously, because of time of exposure, it is likely that genotypes surviving longer are more influenced by environmental factors compared to animals that normally survive less.

From these results, it is clear that homozygous are more severely affected by the disease compared to heterozygous mice, and that when using *Lmna*^{G609G} transgenic mice as an animal model to study the effect of drugs on lifespan it would be optimal to consider sex groups separately. Finally, reproduction and moistened chow, don't affect lifespan in a significant way, and the latter should be considered as a needed refinement method for this model.

4.3.6 Grip Strength test, Open Field test and Numerical Scoring System Specific for *Lmna*^{G609G} Transgenic Mice

Gript Strength test. All mice retained normal grip strength even if they were very debilitated, differently from *Zmpste24*$^{-/-}$ mice (Bergo et al., 2002; Fong et al., 2004). The only difference between genotypes that was noted (registered as a note during the test) was that homozygous at 3 months of age, and heterozygous mice around 8-9 month of age, although remained clinging to the inverted grid, spent more time still compared to both when they were younger and compared to wild type mice. Younger animals of the same genotype and all wild type usually moved fast around the inverted grid, were curious and often looked underneath or groomed themselves remaining anchored to the grid with only 3 paws. Sometimes young animals voluntarily jumped off the grid. Osorio et al. (2011) in the supplementary material of their article, reported that *Lmna*$^{G609G/G609G}$ mice were significantly weaker than both heterozygous and wild type mice when forepaw strength was measured in dynes with a strain gauge sensor. However, such test measured only the forepaw strength of males which can be influenced by size and weight of the animals. The kind of test in our study was the same used for other mice models of progeria (Bergo et al., 2002; Fong et al., 2004; Yang et al., 2006) and therefore the results are comparable to such studies. However, this test might be less sensitive compared to the strain gauge sensor. As stated for *Lmna*$^{HG/+}$, the absence of grip abnormality with the inverted grid test underline that these mice are a faithful model of human HGPS, since children affected do not show significant muscle weakness (Debusk, 1972), differently from what was seen in *Zmpste24*$^{-/-}$ (Bergo et al., 2002; Fong et al., 2004).

The Open Field Test (OFT) is not simply a measure of motor activity but involves other factors such as exploratory drive (curiosity), and fear (anxiety). When placed in the center of the arena mouse typically ran to the walled edge and then explored their way around the whole arena while remaining close to the wall. All the mice had the tendency to spend most of their time in close proximity to the walls of the arena, as can be seen in **Fig. 4.32**. This phenomenon is referred to as thigmotaxis (Gould et al., 2009). Means of vertical activity (VM) for homozygous mice were lower than for wild type or heterozygous mice. Old age groups are reported to exhibit reduction in vertical activity and center time compared with the younger age groups (Shoji et al., 2016). Time spent moving decreased with the age in all groups, with no differences between genotypes (**Fig. 4.32**), indicating that exploratory drive decreases with

age. Center time and activity, together with defecation, in the first 5 minutes, likely measure some aspect of emotionality (Gould et al., 2009). The number of quadrant crossed are reported in **Fig. 4.32** for each genotype and time point considered. Differences were not significant among time points for each genotype, nor among different genotypes at the same age. Studies on age-related changes in behaviour in C57BL/6 revealed an age-dependent decline in locomotor activity (measured as distance travelled in cm) during the early testing period in a novel open field environment (Shoji et al., 2016). Such decrease was associated with increased anxiety-like behaviour with aging rather than a decline in locomotor activity itself (Shoji et al., 2016). In our study mice at the same age even if of different genotypes were exposed to the same stimuli during their whole life, so it is fair to think that anxiety-like behaviours are limited when comparing different genotypes at the same age. However, in order to detect differences between genotype in distance travelled other methods, such as number of infrared beams breaks, which are more precise and accurate, are probably necessary. Furthermore, since high variability is typical in the OFT, a large number of animals should be considered for each group, which was not possible in our study. Freezing was not often seen in animals. However, this is a parameter difficult to detect. Grooming was quite common in all animals at every age (**Fig. 4.32**), confirming, in part, that the bad conditions of the fur were not due to a decrease in the animal self-care.

It was interesting to note that during the 5 minutes during which the OFT was conducted (plus another couple of minutes for weighting the mice and evaluating the health conditions), animals lost between 0 and 0.7 g. Apparently, homozygous lost less weight during the testing. However, it needs to be considered that weight loss is linked to the general weight which is significantly lower in homozygous. In fact, when using the ratio (weight loss/weight)*100 no difference was significant among groups (**Fig. 4.32**).

It has to be kept in mind that despite standardization, OFT tests vary greatly across labs (Crabbe et al., 1999). Thus, experiments characterizing mutants may yield results that are individual to a particular laboratory and also our results are comparable intra but not interlab. Moreover, considering the high variability between individuals, groups should be numerous. Till date, we had the chance to consider only 3 homozygous mice in our analysis, which is a far too small group to use for statistical analysis for OFT. Furthermore, tests are still being conducted and yet we haven't gathered data for OFT of older heterozygous and wild type mice. Nevertheless, conducing the OFT we got the idea that *Lmna*^{G609G} transgenic are active,

lively and social till the very end of their life when their health condition worseness very quickly. Heterozygous mice at 6 months of age and homozygous mice at 3 months of age are smaller than wild type littermates, they weigh less, have marked kyphosis, show premature aging fur but they still act in a similar way to their siblings.

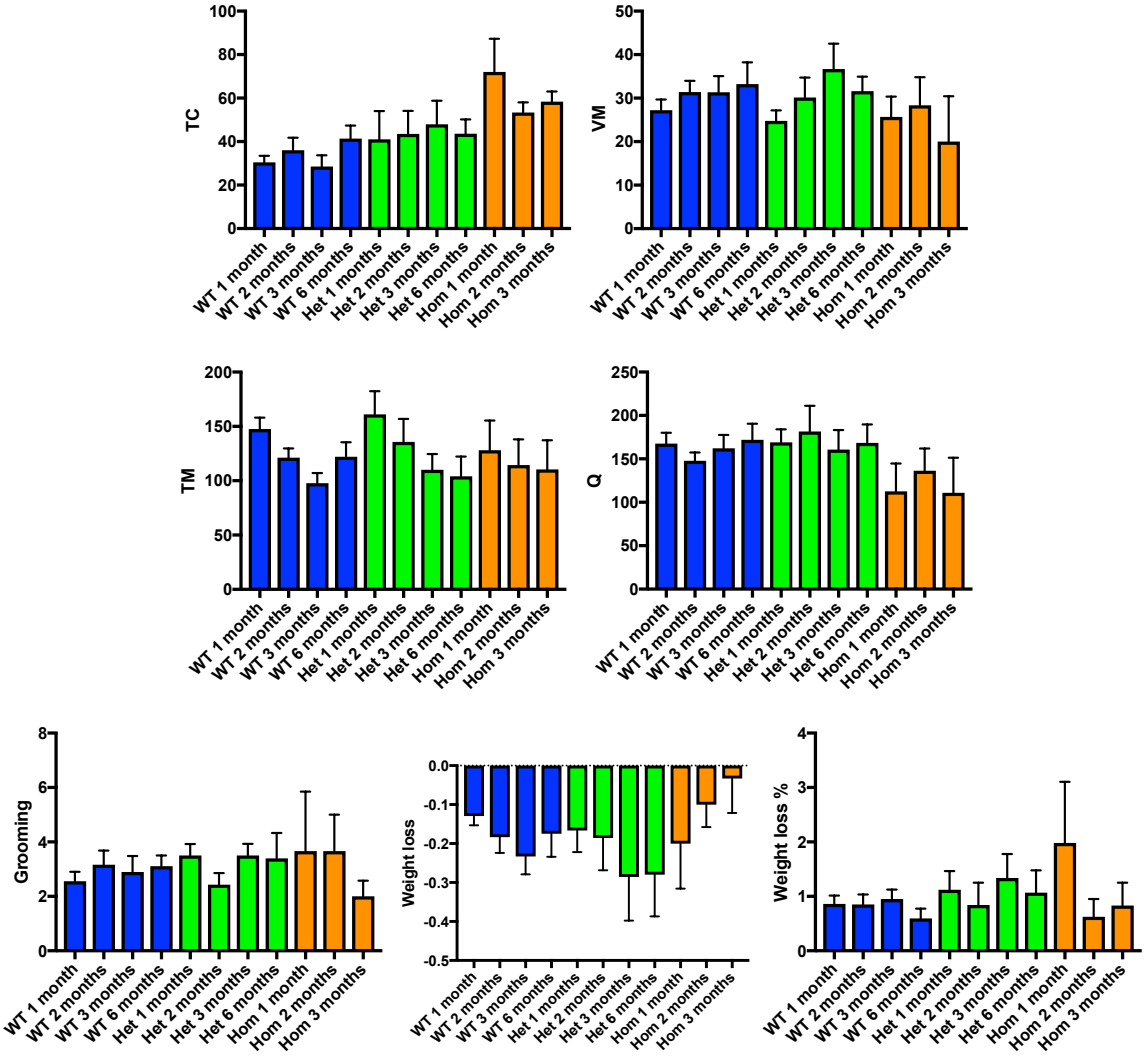


Fig. 4.32*: Bars and boxes graphs representing wild type (WT) mice at 1 (n=17), 2 (n=17), 3 (n=17), 6 (n=9) months of age, heterozygous (Het) mice a 1 (n=8), 2 (n=7), 3 (n=6), 6 (n=5) months of age, and homozygous (Hom) at 1 (n=3), 2 (n=3) and 3 (n=3) months of age. TC= Time spent at the center; VM= Vertical movements; TM= Time spent moving; Q= Numbers of quadrant crossed. Time is expressed in seconds.*

Objective indices of phenotypic alterations can be useful in following the progression of a disease. Based on this consideration we proposed a numerical scoring system (NSS) specific for *Lmna*^{G609G} transgenic mice that gives values (from the best to the worst condition possible, 3 to 10) considering parameters such as fur, gait and activity (**Table 4.1**). This assessment revealed to be fast and easy to use. As it can be seen from the scores given after each OFT

(**Fig.4.33**), wild type mice had a nice fur, were active and walked normally until the last observation (6 months of age). This was not true for transgenic mice. *Lmna*$^{G609G/+}$ did not show much difference from wild type mice up to 3 months of age. At 6 months, the NSS was significantly higher in *Lmna*$^{G609G/+}$ ($p = 0.0249$). The final score was influenced especially by the fur and skin conditions. However, at 6 months of age they were still very active. *Lmna*$^{G609G/G609G}$ were more severely affected and at 3 months of age already reached a mean score of 6. Again, the final score was influenced especially by the fur and skin conditions.

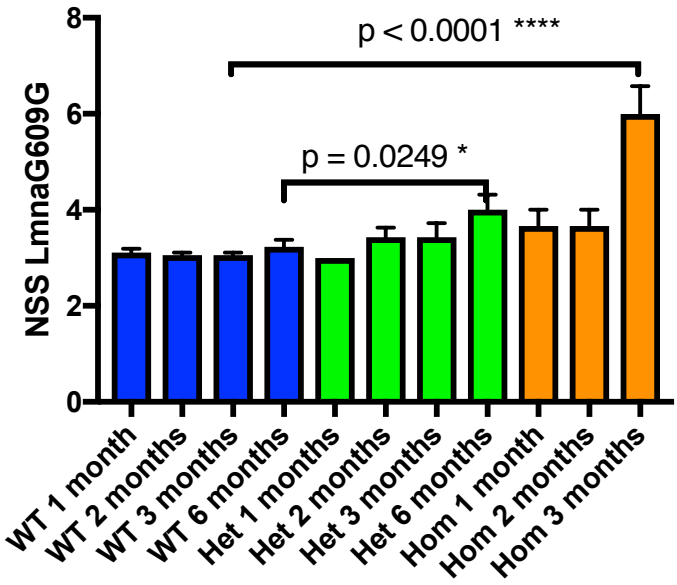


***Fig. 4.33**: Numerical scoring system used to describe the health conditions of Lmna*$^{+/+}$*, Lmna*$^{G609G/+}$ *at 1, 2, 3 and 6 months of age and of Lmna*$^{G609G/G609G}$ *at 1, 2 and 3 months of age.*

4.3.7 Radiological Examinations

The acquisition of an abnormal posture (hunched-up) and kyphosis characterized this mouse model of progeria, as already reported by Osorio et al. (2011). Kyphosis was also observed by *in vivo* clinical observations (**Fig. 4.20**), and during necroscopy. The seriousness and the onset of these features depended on age and genotype (**Fig. 4.34**).

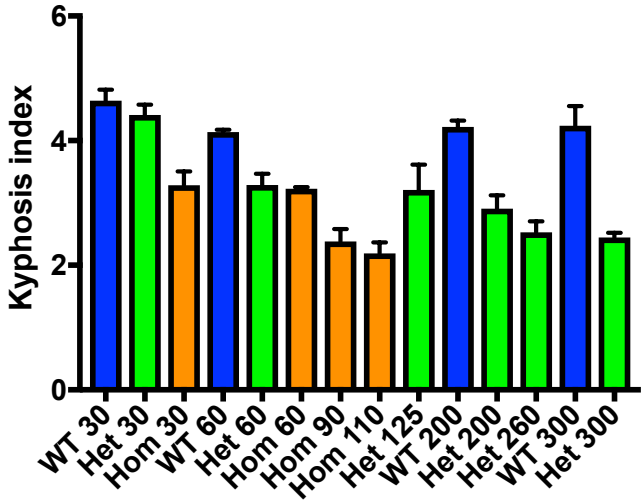
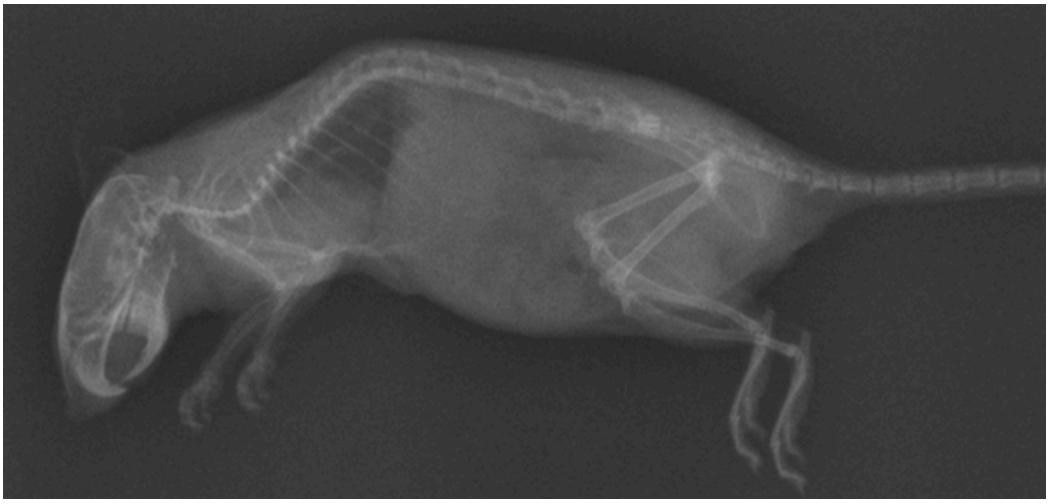


***Fig. 4.34**: Kyphosis index for wild type mice (WT), heterozygous (Het) and homozygous (Hom) mice, at different ages. Severity of kyphosis depends on age and genotype.*

No wild type animals had relevant alterations that could be seen with the X-rays up to 300 days of age, KI means were always above 4, and incisors were always normal (**Fig. 4.35**).



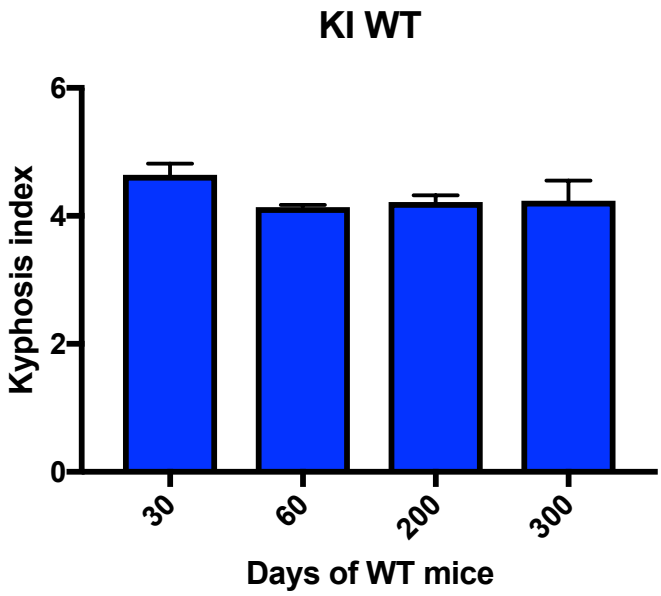


Fig. 4.35: *X-rays of a wild type mice at 300 days of age (above). Note the normal anatomy of the mouth and disposition of the incisors. Abundant adipose tissue. Kyphosis indexes of wild type mice at different ages (below).*

KI indexes of the heterozygous mice at weaning (30 days) had no significant differences compared to wild type mice (**Fig. 4.36**), differently from homozygous.

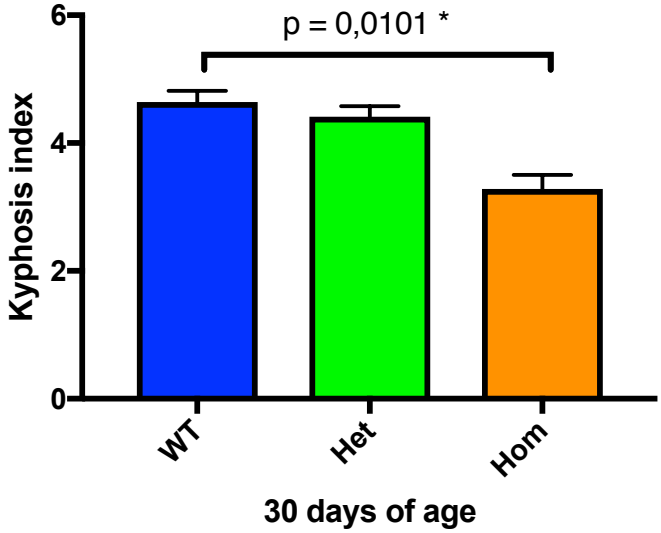


Fig. 4.36*: KI of wild type mice (n=4), heterozygous (n=5) and homozygous (n=3) Lmna^{G609G} transgenic mice at 30 days of age.*

Kyphosis index for heterozygous mice was significantly lower than wild type mice starting from 200 days of age (p = 0.0152), although it might have been detectable earlier if we had had wild type controls for heterozygous at 125 days. The KI progressively got worst throughout the life of heterozygous as it can be seen in **Fig. 4.37.** In particular, during the last months of life this

condition was extremely amplified (**Fig. 4.38**). The spinal scoliosis was always at the level of the chest stretch.

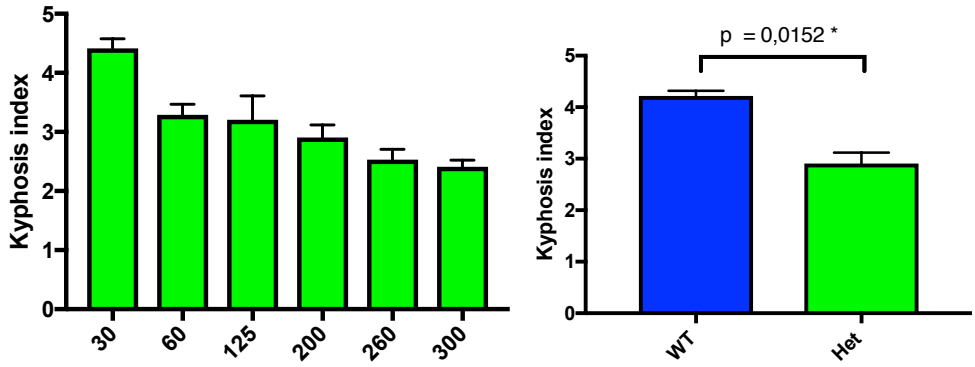


***Fig. 4.37**: KI for heterozygous mice at 30, 60, 125, 200, 260 and 300 days of age (left). Kyphosis gets worst with age. Comparison between KI of wild type mice and heterozygous at 200 days of age (right).*

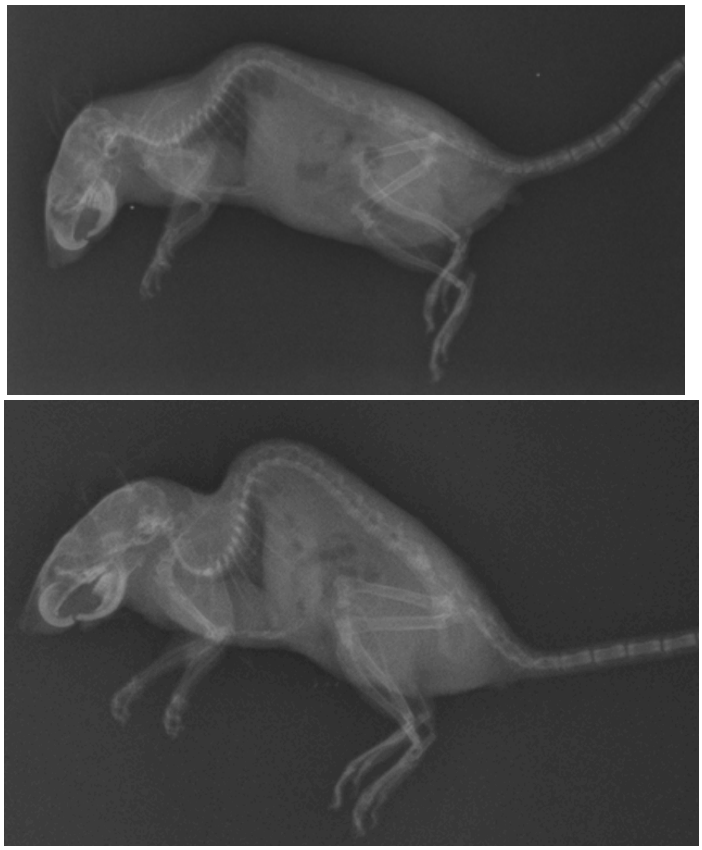


***Fig. 4.38**: X-rays of the same heterozygous mice at 300 days (above) and 336 days (below). KI index went in 36 days from 2.52 to 1.60.*

Also, abnormalities of the incisors were common (**Fig. 4.39**).

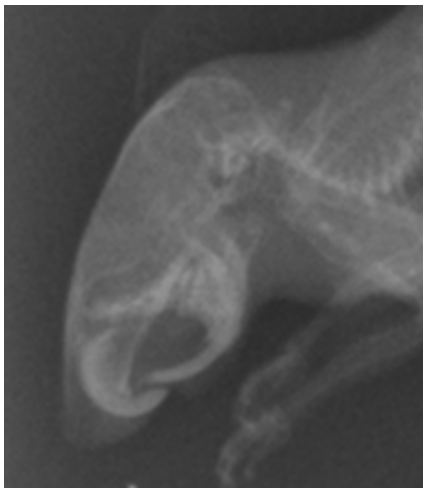


Fig. 4.39*: Heterozygous mice at 260 days of age showing abnormalities in the incisors. Lower incisors grow within the palate because of malocclusion.*

Homozygous mice at 30 days of age already had a lower KI compared to wild type mice (**Fig. 4.36**). This difference was significant and got progressively worst in a short amount of time (**Fig. 4.40** and **Fig. 4.41**). Also, lower incisors presented progressively a more flattened profile while the upper incisors showed an abnormal curvature with consequent malocclusion (**Fig. 4.41**). Moderate reduction of the abdominal fat was also evident compared to wild type mice of the same sex and age.

No differences in kyphosis nor in the skull were evidenced between sexes of same genotype and age.

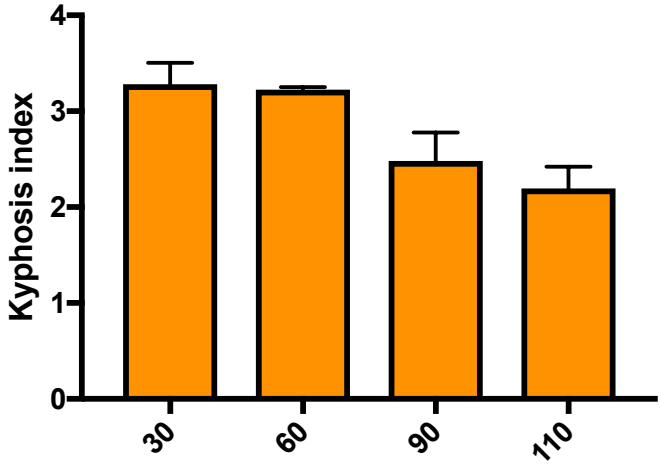


Fig. 4.40*: KI for homozygous mice at 30, 60, 90 and 110 days of age. Kyphosis got worst with age.*

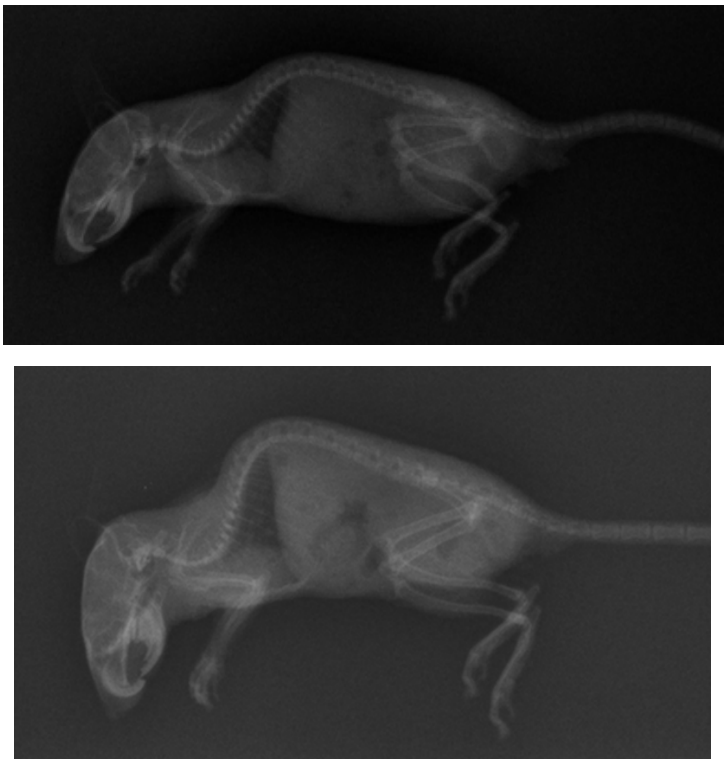


Fig. 4.41*: Same homozygous mouse at 40 days (above) and at 90 days (below). KI index went from 3.03 to 2.18. Note how at 90 days (below) malocclusion is present.*

Comparison between the wild type mice, the heterozygous and homozygous mice can be seen in **Fig. 4.42**.

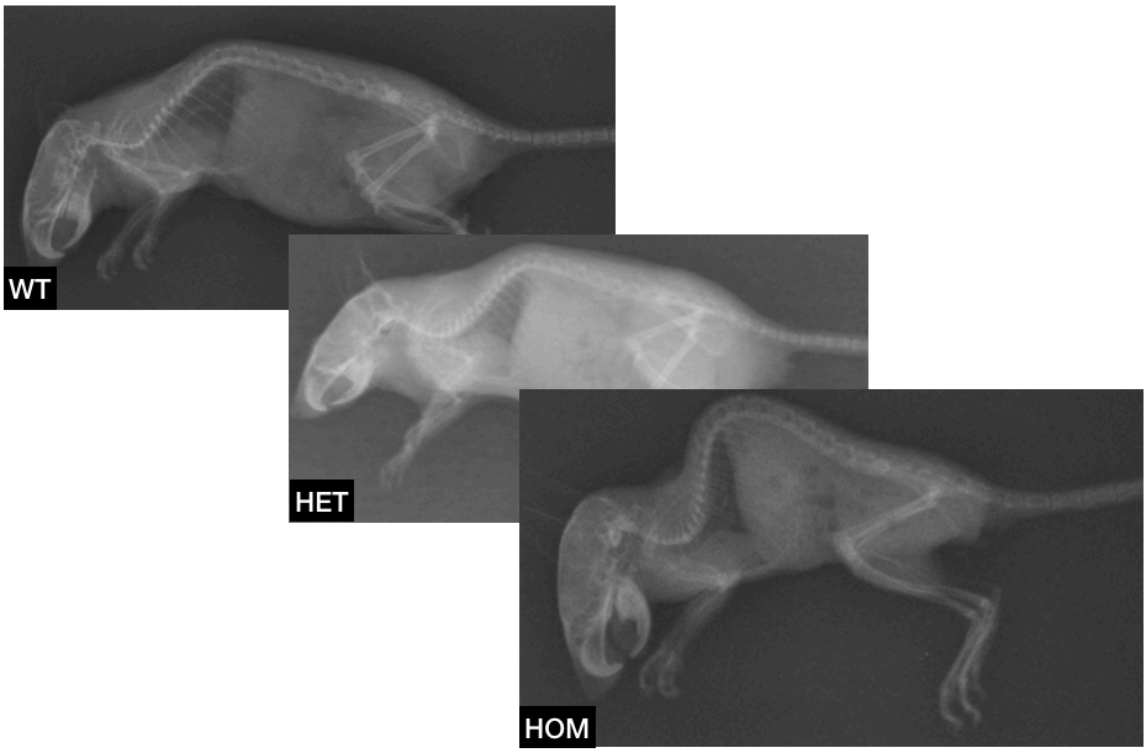


Fig. 4.42: *Comparison between a wild type (WT) mouse at 170 days, and heterozygous (HET) at 125 days and a homozygous (HOM) at 110 days.*

Kyphosis arising in human normal aging is often related to osteoporosis through deformity of the vertebral bodies (Cummings & Melton, 2002), or to annulus degeneration (Resnick, 2002). Furthermore, in humans with conditions that lead to a reduction of vertebral support could result in thoracolumbar deviation in a ventral or dorsal plane (lordosis or kyphosis, respectively) or a lateral deviation of the spine due to the effect of gravity (scoliosis) (Laws & Hoey, 2004). In mice, due to the quadrupedal gait, conditions such as neuromuscular weakness would result in development of only kyphosis (Laws & Hoey, 2004). Human patients with HGPS often present scoliosis and cervicothoracic kyphosis (Chawla et al., 1986; Monu et al., 1990; Rodriguez et al., 1999; Hennekam, 2006). The early onset of spinal deformity in *Lmna*^{G609G} transgenic mice could be attributable to osteoporosis, being causally similar to what happens in normal aging. However, in several transgenic mice, kyphosis was linked to osteosclerosis (Dabovic et al., 2002), growth plate abnormalities (Iba et al., 2001), and muscular dystrophy (Burkin et al., 2001), compression deformities, wedging or fractures. In our study, radiographs were not of sufficient resolution to allow the evaluation of such changes that cannot therefore be excluded.

4.3.8 Pathological/Histological Observations

Tissues were surveyed at autopsy and by microscopic analysis. No consistent gross pathology was found in the external ear, skin, brain, testis, ovary, skeletal muscle, bone, liver, spleen, kidney, or heart for both genotypes of *Lmna*^{G609G} transgenic mice. However, some alterations were registered confirming the *in vivo* observations, such as the size reduction, the moderate to severe kyphosis in the first thoracic vertebrae, a generalized loss of fat deposits, the presence of alopecic areas and dental malocclusion for all *Lmna*^{G609G} transgenic mice.

Kyphosis was never observed in wild type mice (0/7), as also for skin alterations (0/7) (**Fig. 4.43A** and **B**). However, when the grading system was applied on 7 wild type three were graded 1, three graded 2 and one graded 3.

In one case there was a moderate hypoplasia of the lymphoid tissue and expansion due to a round cell tumour. In this case there was also hypoplasia of the adipose tissue of the subcutis.

No appreciable alterations at the level of the aorta were observed (0/4)(**Fig. 4.43C**). They were all graded 1 using the specific grading system.

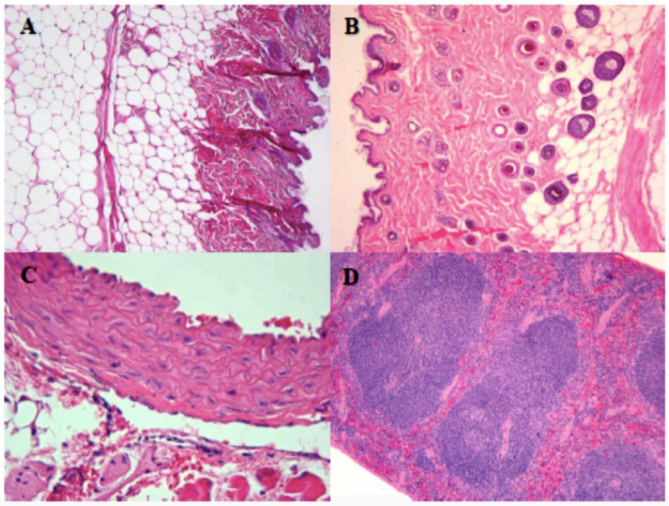


***Fig. 4.43**: H&E staining of skin **(A, B)**, aorta **(C)** and spleen **(D)** from wild-type mice. Note normotrophic and normoplasic skin with well-developed hair follicles and subcutaneous fat (grade 1) **(A, B)**. No appreciable alterations at the level of the aortic wall **(C)** which shows a good cellularity (grade 1). Normotrophic spleen **(D)**. (Anatomo-Pathological Service of DIMEVET).*

Twenty-nine *Lmna*$^{G609G/+}$ transgenic mice were examined. They all suffered from kyphosis. In all animals, alopecia associated with reduction in number of follicles (mainly in the catagen phase) was observed, as also dermal fibrosis. Using the grading system two heterozygotes were graded 2, eight graded 3 and nineteen graded 4 (**Fig. 4.44A**).

The alterations at the level of the aortic wall were frequent (21/24) with a reduction in the cell number, in the thickness of the tunica media and a multifocal accumulation of weakly basophil material likely attributable to mucopolysaccharides (**Fig. 4.44C**). When the grading system was used in heterozygotes three arteries resulted graded 1, two graded 2, fourteen graded 3 and five graded 4.

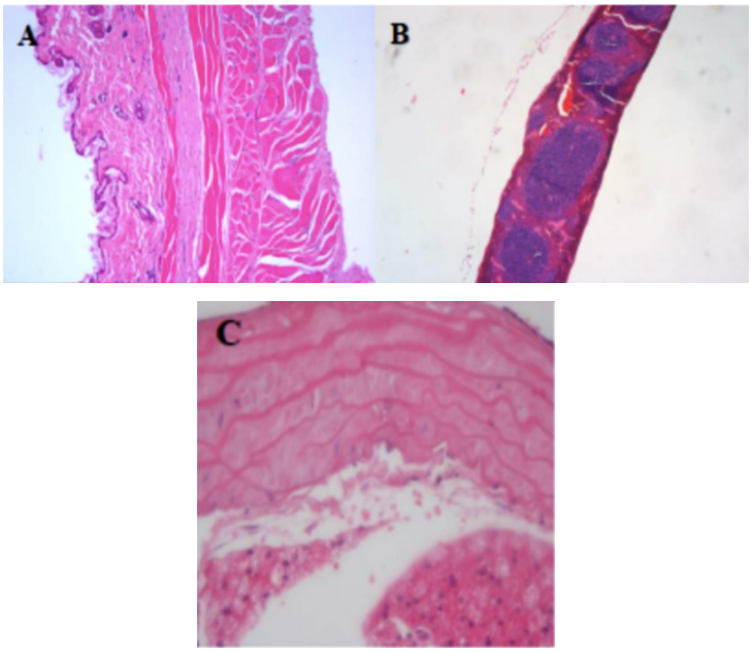


Fig. 4.44*: H&E staining of the abdominal skin* ***(A)****, spleen* ***(B)*** *and aorta* ***(C)*** *from an heterozygous mice. Note the atrophy of hair follicles and the absence of subcutaneous fat* ***(A)*** *(grade 4), the hypotrophy of the spleen* ***(B)*** *and the cell reduction and a slight myxoid degeneration at the level of the aortic wall* ***(C)*** *(grade 4) (Pathological Service of DIMEVET).*

Nine *Lmna*$^{G609G/G609G}$ transgenic mice were examined. All of them suffered from kyphosis. Alopecia associated with reduction in number of follicles and dermal fibrosis was seen for 8/9 subjects. Two animals were graded 2, three graded 3 and four graded 4 (**Fig. 4.45**). The subcutaneous fat was completely missing in all animals. The alterations at the level of the aortic wall were present in all animals. Seven aortas were available, two of which were graded 2, two graded 3 and three graded 4 (**Fig. 4.45**).

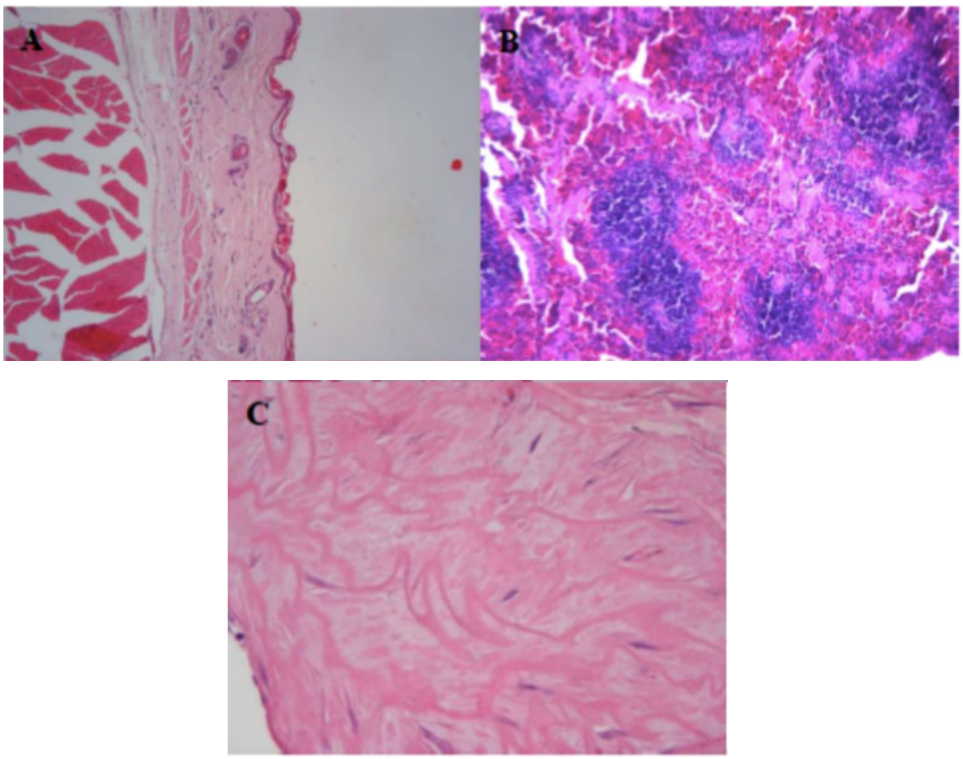


***Fig. 4.45**: H&E staining of the skin **(A)**, spleen **(B)** and aorta **(C)** from an homozygous mice. Note the atrophy of adipose tissue and the disappearance of hair follicles **(A)** (grade 4), the hypotrophy of the lymphoid tissue in the spleen **(B)**, the cell reduction and a myxoid degeneration at the level of the aortic wall **(C)** (grade 3) (Pathological Service of DIMEVET).*

The histochemistry of the aorta revealed that the basophilic material seen with H&E was composed by non-sulphated mucins and not by sulphated mucins or glicoproteins (**Fig. 4.46**).

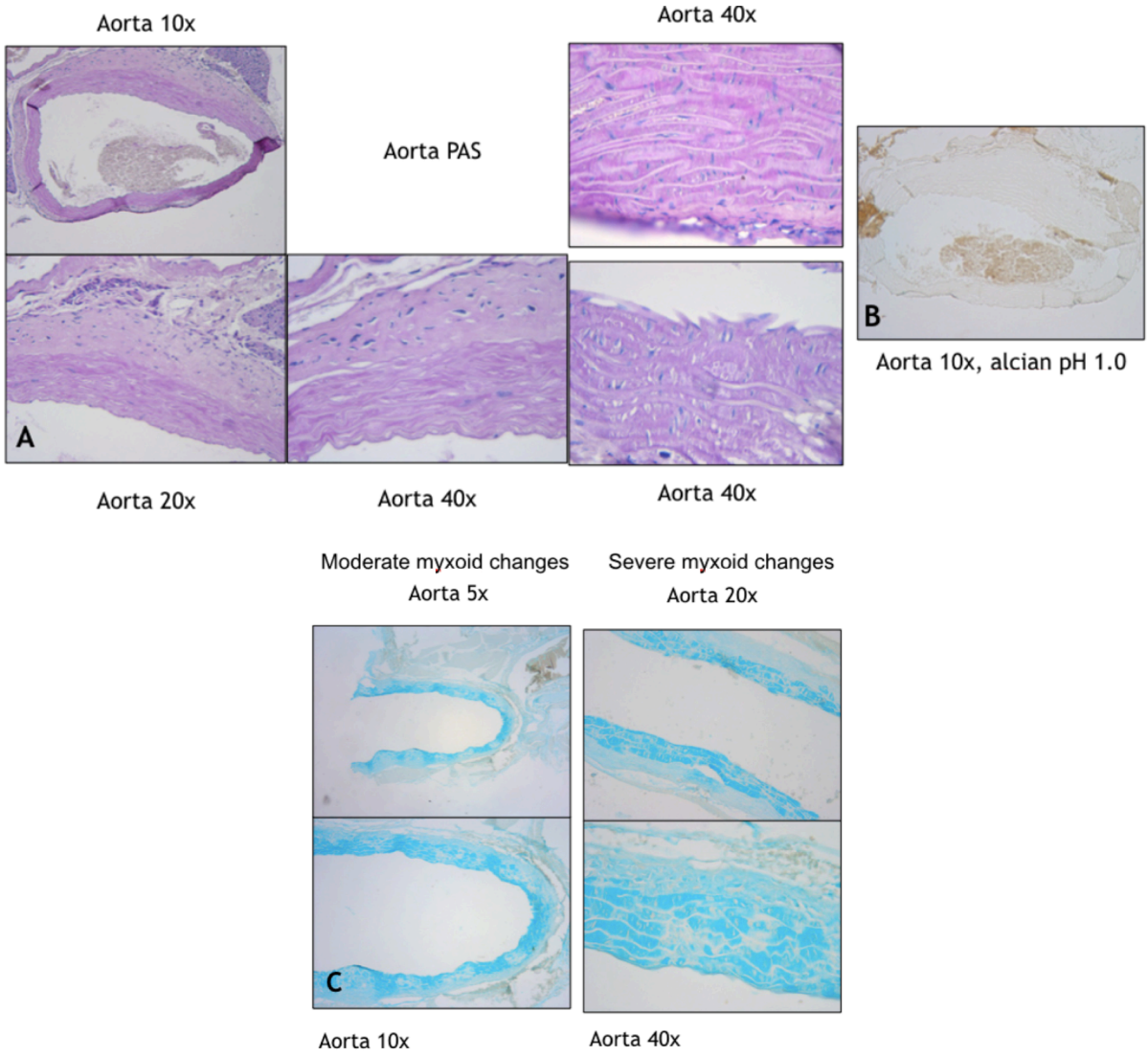


***Fig. 4.46**: Aorta stained with **A)** PAS, negative for glicoproteins; **B)** alcian pH 1, negative for sulphated mucins and GAG; **C)** alcian pH 2.5, positive for non-sulphated mucins (Anatomo-Pathological Service of DIMEVET).*

The grading system for skin and aorta lesions revealed significant differences ($p < 0.05$) between both genotypes of transgenic animals and *Lmna*$^{+/+}$, but not between *Lmna*$^{G609G/+}$ and *Lmna*$^{G609G/G609G}$ (**Fig. 4.47**).

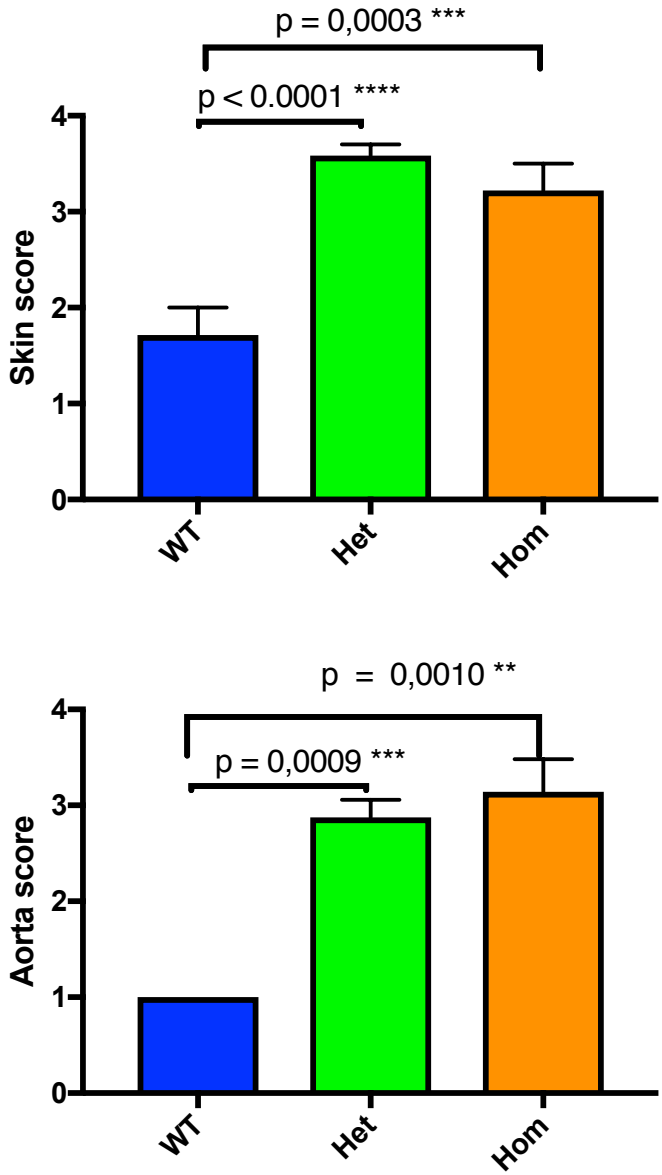


Fig. 4.47: *Skin (above) and aorta (below) grading scores of transgenic animals who were sacrificed because they had reached the humane endpoint, and of wild type mice at the same age (controls). Differences between transgenic and wild type mice were significant, but not between heterozygous and homozygous mice.*

Important cardiovascular alterations in terms of vascular smooth muscle cells at the level of the medial layer of the aortic arch were also found in *Lmna*$^{G609G/G609G}$ transgenic mice from Osorio et al. (2011), together with alteration of depolarization of the heart ventricular wall. However, these alterations were not graded nor evaluated for heterozygous mice. Villa-Bellosta et al. (2013) found an important vascular calcification in *Lmna*^{G609G} mice revealed by

planimetric analysis of Alizarin Red–stained aortic cross sections. Such calcification was linked to a reduced extracellular accumulation of pyrophosphate resulting from the increased tissue-nonspecific alkaline phosphatase activity and the diminished ATP availability caused by mitochondrial dysfunction in vascular smooth muscle cells (Villa-Bellosta et al., 2013).

Premature death of *Lmna*^{G609G} transgenic mice could be linked to the cardiovascular alterations found, which also occur in HGPS patients and during normal aging. However, other causes, such as malnutrition, cannot be excluded.

Interstitial pneumonia (5/7 wild type; 22/27 heterozygotes; 7/8 homozygotes) and hypoplasia/atrophy of the spleen lymphoid tissue (1/7 wild type; 6/24 heterozygotes; 2/6 homozygotes) (**Fig. 4.44B** and **4.45B**) were non-specific changes observed in all genotypes. However, the use of sterile caging for this mouse model is highly recommended and would represent a preventive strategy useful for future studies using *Lmna*^{G609G} transgenic mice.

4.3.9 µCT Analysis

Individual bony elements are conserved between mouse and human skulls (**Fig. 4.48**). Analysis of the craniofacial skeleton of *Lmna*^{G609G} transgenic mice using µCT scans helps establishing parallels in human and mice phenotypes resulting from the same genetic alteration.

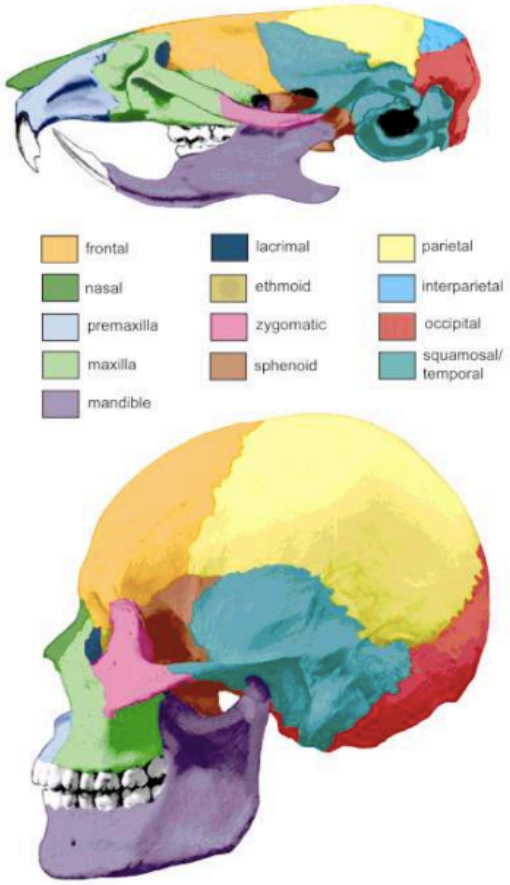


Fig. 4.48: *Mouse and human skulls. Color-coding shows correspondence of structures between the species. The interparietal bone (bright blue on the mouse skull) is an example of a skull bone that exists in the more primitive (mouse) form, but not in the more derived human skull (Ritchsmeier et al., 2000; reproduction authorized).*

Transgenic mice had an abnormal skull shape. The bone reliefs were less marked and the indentations of the sutures between cranial bones were morphologically reduced in heterozygous and homozygous mice in relation to wild type ones (**Fig. 4.49**). Transgenic mice also had lower micrognathia (small mandible) causing malocclusion. The consequence of such micrognathia is that mutant mice regularly showed incisors anomalies in both implantation and morphology of the incisors. In particular, upper incisors had an increase of the curvature (reduced radius). The lower incisors of wild type mice were triangular in shape when

dissected, whereas the ones of mutant mice were cylindrical and showed a more flattened profile (**Fig. 4.49**).

Human HGPS patients normally have a relatively large neurocranium compared to the viscerocranium (especially the mandible) (Hennekam, 2006), similarly to what can be seen in *Lmna*^{G609G} transgenic mice. In particular, in humans the chin has a normal size and shape and becomes smaller after 1–2 years. There is osteolysis of the viscerocranium, but the osteolysis of the mandible is more marked and causes retrognathia. Both the horizontal and ascending rami become smaller with age, and the mandibular angle increases (often to about 150 degrees) (Hennekam, 2006). The decrease in size of the maxilla and mandible causes crowded teeth (Hennekam, 2006) in parallel to what it could be seen in transgenic mice.

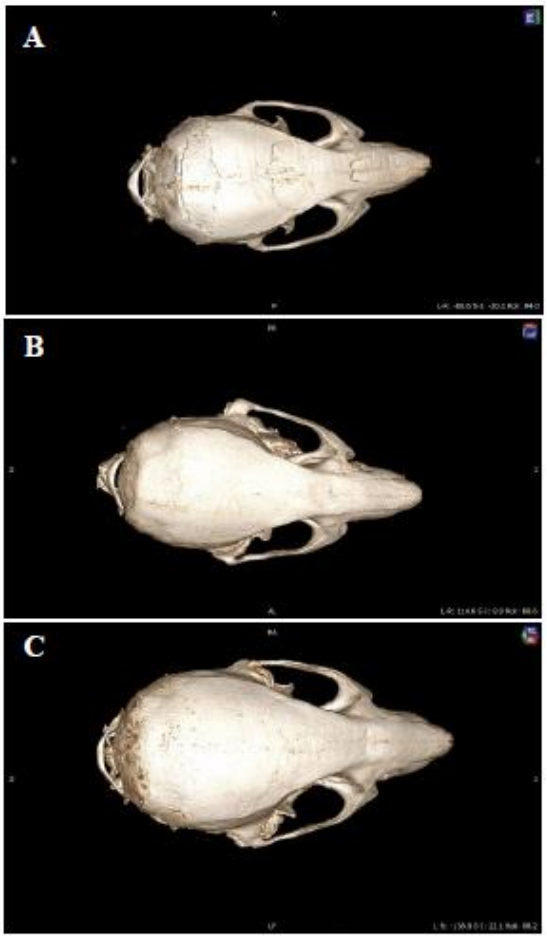


Fig. 4.49*: Top view of skulls. Surface renderings of µCT of the skull of wild-tipe* ***(A),*** *heterozygous* ***(B)*** *and homozygous* ***(C)*** *mice. The bone reliefs and the sutures between cranial bones were less marked in heterozygous and homozygous mouse compared to wild-type (Laboratory of Medical Technology Rizzoli Orthopaedic Institute, Bologna).*

Moreover, in the lateral view of the skull µCT scans showed absence of a small segment in the left zygomatic arch of homozygous mouse (**Fig. 4.50**). Osteolytic lesions of the zygomatic arch

were also evidenced in another mouse model of progeria (Yang et al., 2006). Some form of osteolysis is invariably present in any human patient with HGPS and can be found also at the mandible, neurocranium, and viscerocranium (Hennekam, 2006).

The skull of homozygous mice was smaller than that of their wild type counterparts, however they were not microcephalic since skull dimensions were proportional to their reduced body dimension and weight, contrary to what reported by de Carlos et al. (2008) in another progeroid model. Human patients are not microcephalic and usually vault appears bigger than the face only because of the decrease in viscerocranium size (neurocranium remains near normal) (Hennekam, 2006).

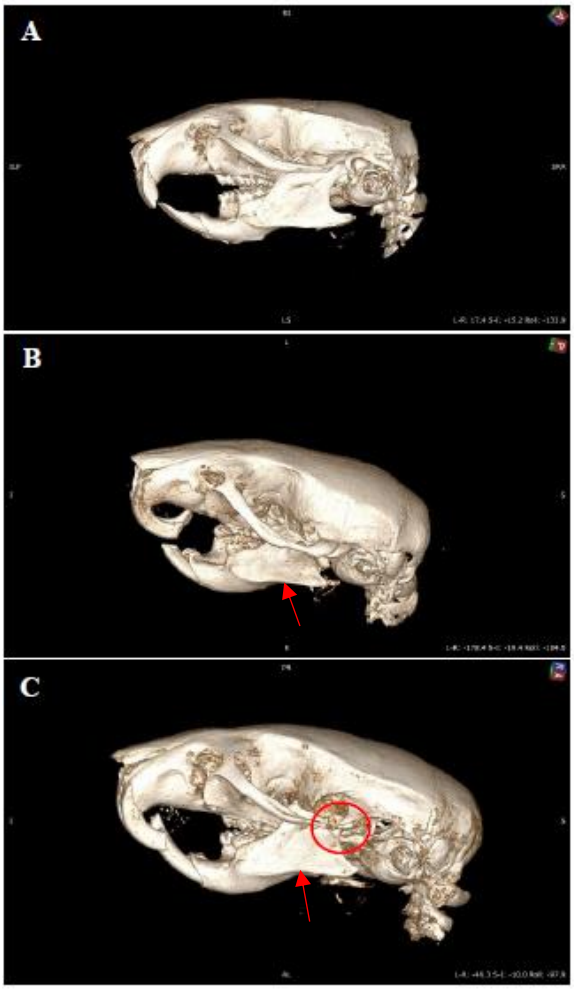


Fig. 4.50. *Lateral view of skull. µCT of the skull of wild-type* ***(A),*** *heterozygous* ***(B****) and homozygous* ***(C)*** *mice. Micrognathia in both transgenic mice* ***(B, C)*** *is indicated with the red arrows. Micrognathia caused dental malocclusion. The red circle in the* $Lmna^{G609G/G609G}$ ***(C)*** *mice indicates an osteolytic lesion of the zygomatic arch (Laboratory of Medical Technology Rizzoli Orthopaedic Institute, Bologna).*

4.4 Conclusions

Detailed phenotypic data are being increasingly incorporated into studies and are particularly important when investigating longevity and comorbidities associated with aging.

Because genome maintenance mechanisms are generally highly conserved between species, mice can represent valuable models to study genetic alterations, pathways and possible treatments to address certain aspects of aging (Gurkar & Niedernhofer, 2015).

As seen in **Chapter 3** (**Paragraph 3.9**), mouse models intended to mimic human HGPS have not always yielded precisely the anticipated phenotype, reflecting the complexity of this disorder and the underlying mechanisms. Osorio et al. (2011) engineered on a well-known genetic background (C57BL/6) a mouse model with the same genetic mutation of human HGPS and gave a first description of transgenic *Lmna*^{G609G} mice, concentrating on the homozygous mice.

Our first goal was to define in detail the phenotype and breeding characteristics of *Lmna*^{G609G} transgenic mice. Both the homozygous and heterozygous were considered. This study represents the first description of this model over a long period (2 years), with special attention to reproduction, weaning, growth and expression of specific symptoms. Homozygous mice for the mutation were born with a lower frequency compared to what expected by Mendelian's proportion, they were not sterile but could not successfully deliver pups. Fortunately, heterozygous mice could be mated together, females being good breeders such as wild type mice. This mouse model not only shares the same genetic cause of human HGPS, but in this study, we observed that both genotypes (homozygous and heterozygous) manifest most of the described human signs and symptoms (**Table 4.5**). There are many similarities between the disease of interest in humans and the animal model: shorter life expectancy, reduced body growth, hair loss, absence of subcutaneous fat, dry skin, decreased mobility, skeletal and cardiovascular problems.

It has been stated that homozygous mice represent a better model of human HGPS than *Lmna*$^{G609G/+}$ heterozygotes (Osorio et al., 2011). However, in our opinion, this depends on the aim of the research. For example, homozygotes live around 108 days and show signs of the disease really soon after weaning, making it difficult to test the efficacy and the safety of chronic treatments starting early in life, before symptoms may appear. Therefore, when evaluating chronic treatments heterozygous mice could be more appropriate models.

Furthermore, if not only lifespan but also quality of life is taken into consideration, it might be easier to follow progression of signs and symptoms which occur more slowly in heterozygous.

Feature	Human HGPS	*Lmna*^{G609G} transgenic mice
Growth	Sever growth deficiency. Kids are shorter and weight less	Severe growth deficiency in homozygotes, moderate in heterozygotes. Mice are smaller in size and weight
Hair/Coat alterations	Balding, downy hair with the tendency to curl, absence of eyebrows and eyelashes, scarce or absent body hairs	Periocular alopecia and thin coat. Opaque and rough hair. Fur prematurely has grey strikes
Ocular alterations	Not reported	Microphthalmia, anophthalmia, opacity of the cornea more frequently observed in homozygotes then in heterozygous. However, these alterations should be further investigated because they might be connected to the C57BL/6 background
Skin alterations	Moderate scleroderma; thick, thin, dry and atrophic skin; oedema	Dry, thin and with erythema-like redness
Lipodystrophy	Expressed	Expressed
Cardiovascular alterations	Expressed	Expressed
Mobility	Decreased Cervicothoracic kyphosis, scoliosis Shuffling gait	Decreased Cervicothoracic kyphosis Shuffling gait
Osseous apparatus		
Acra	Osteolysis of the distal phalanges; nails dystrophia	Nails dystrophia
Clavicles	Narrow shoulders	Undetectable
Mandible	Retrognathia	Reduced and flattened mandible Micrognathia
Viscerocranium	Small; decreased size of the maxilla and mandible with crowding of teeth	Micrognathia with dental malocclusion
Neurocranium	Mild; normal size dependent on the growth of the brain; vault relatively large compared to the face; delay in cranial suture closure	Normal skull size. Bone reliefs and the sutures between cranial bones are less marked
Lifespan	13 years of age	108 days homozygotes* 287 days heterozygotes* **significant differences among sex groups*
Ratio females:males affected	1:1.5 (only heterozygous)	1:1.13 (homozygous) 1:1.53 (heterozygous)

***Table 4.5**: Comparison between G609G transgenic mice and human HGPS. HGPS patients are all heterozygotes Lmna$^{G608G/+}$. Mice are more tolerant than humans to accumulation of prelamin A forms and both heterozygous and homozygous survive. However, although the signs and symptoms are the same in heterozygous and homozygous mice, Lmna$^{G609G/G609G}$ are affected earlier in life compared to Lmna$^{G609G/+}$.*

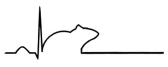
Furthermore, we evidenced a significant difference between females and males in terms of weight trends and lifespan. In researches using this animal model, such differences should be kept in mind and groups of tested animals should be constructed accordingly.

Human patients commonly have a lifespan of 13 years, strokes being the first cause of death. In mice, important alterations have been evidenced at the thoracic aorta level, but it is hard to establish if this could be the spontaneous cause of death. In fact, in parallel with the worsen of the signs, animals result malnourished and eventually they had to be euthanized because for this reason they reached the humane endpoint.

Our description will help other research groups in choosing breeding strategies and mice housing, together with helping to maintain the focus on the signs and symptoms described as most characteristic of the mouse model and eliminating confounding signs deriving from the C57BL/6 genetic background, such as eye defects. The outcomes of this study should represent, together with previously published data, the starting point for planning future researches on preclinical treatment trials. The study of this animal model of progeria allowed to find out how much the murine model pathological features resemble phenotypically the human disease, as well as to define to which extent this mutation influences all aspects of breeding these animals.

Till date, this is the best animal model for the study of pathway mechanisms and a rapid model in preclinical studies to treat HGPS. In regard to natural aging, as other mice models, *Lmna*^{G609G} transgenic mice are not perfect models. Generally, mice models of accelerated aging are generated to ask specific questions about the physiological function of a protein (Baker et al., 2004; Niedernhofer et al., 2006) or to test a specific hypothesis about the contribution of particular type of damage to aging (Kujoth et al., 2005; Trifunovic et al., 2004). There is often incomplete overlap between the histopathologic lesions seen in rapidly aging mice and those commonly associated with normal aging indicating that models are segmental or tissue specific. However, the mice that were generated to model a human progeroid syndrome have translational potential (Chen et al., 2013; Niedernhofer et al., 2006) for discovering therapeutics for rare diseases and potentially aging in the general population (Gurkar & Niedernhofer, 2015). Moreover, aging results from a complex interplay between genetics and the stresses placed on it by its particular environment. The genetic background of mice has an important role in modifying the penetrance and expressing of clinical manifestations of DNA repair deficiencies. Environmental factors, including housing conditions, infectious agents,

and diet likely play a role in the expression of aging phenotypes in mouse models, and perhaps also in humans (Collis & Tabak, 2014; Gurkar & Niedernhofer, 2015).



Chapter 5

Treatment in *Lmna*^{G609G} Transgenic Mice

Preliminary testing

5.1 Objective

The bases of this preliminary research can be found in one main published article (Pellegrini et al., 2015). Such article reported that all-trans retinoic acid (ATRA) acted synergistically with low-dosage rapamycin (RAPA) reducing both progerin and prelamin A and increasing the lamin A to progerin ratio in human's HGPS fibroblast.

The aim of this preliminary study, was to assess the validity of the model evaluating the effect of *in vivo* treatment with ATRA-RAPA at low dosages using both homozygous and heterozygous *Lmna*^{G609G} transgenic mice. The desired effect was to extend lifespan of treated animals, and, secondarily, to improve the disease phenotype.

5.2 Materials and Methods

5.2.1 Animal Model and *In Vivo* Techniques

Facilities, housing and breeding, animal model and techniques are the same as the ones described in **Chapter 4**.

The animals used to study the efficacy of ATRA+RAPA treatment were chosen within the up-above described colony. Only male mice were used, and in particular, 10 heterozygous (*Lmna*$^{G609G/+}$) mice and 10 homozygous (*Lmna*$^{G609G/G609G}$) mice were selected for this study.

5.2.2 Treatment

Mice were treated twice weekly (after weight recording) by intraperitoneal injection (IP). Mice were held by the nape of the neck and extended by pressing the tail to the palm of the hand. They were tilt with the head slightly towards the ground (head lower than the hind end) in order to allow the abdominal viscera to shift cranially and minimize accidental puncture of abdominal organs at site of injection. The abdominal wall was penetrated in a line parallel with the mouse backbone and at an approximate 30-40° angle to the abdominal wall (Miner et al., 1969). The lower quadrants are posterior to a line connecting the anterior junction of the hind legs to the body. The stomach, cecum and bladder, important targets for misplaced injections, lie on the left side of the peritoneal cavity; for this reason, we preferred the lower right quadrant to the lower left quadrant (**Fig. 5.1**). Syringe of 1 mL were used with 26 G needle Gauge. The treatment was injected after reaching room temperature.

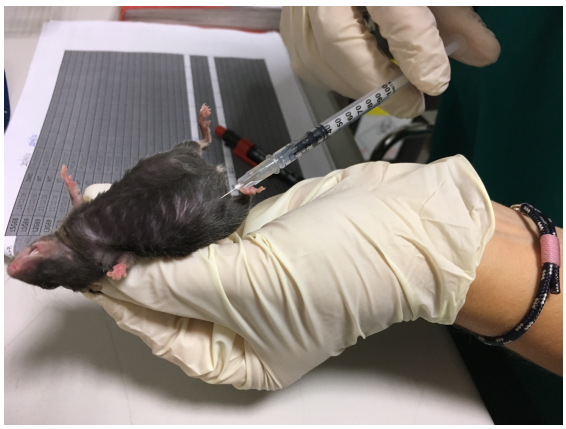


***Fig. 5.1**: Intraperitoneal injection.*

Rapamycin and all-trans retinoic acid treatment (ATRA+RAPA) was prepared at the Institute of Molecular Genetics CNR, Unit of Bologna. Aliquots were prepared (2 mL vials) and properly stored at -20°C for not more than 3 months. Freeze-thaw cycles were avoided and aliquots were defrosted, brought at room temperature and vortexed prior to the injection.
The combination of the two drugs (ATRA+RAPA) justified the lower dosage in comparison to the ones found in literature (Woodrum et al., 2010; Kwok et al., 2012). The dose regimen used in such study was 1 mg/kg of rapamycin and 0.4 mg/kg of all-trans retinoic acid, twice weekly.

Study 1. ATRA+RAPA *Lmna*$^{G609G/+}$

Five *Lmna*$^{G609G/+}$ male mice received ATRA+RAPA starting from 6 weeks of age and throughout their whole life. Five mice of the same genotype and sex were used as negative controls for this treatment. Animals were euthanized when they reached the humane endpoint.

Study 2. ATRA+RAPA *Lmna*$^{G609G/G609G}$

Five *Lmna*$^{G609G/G609G}$ male mice received ATRA+RAPA starting from 6 weeks of age and throughout their whole life. Five mice of the same genotype were used as negative controls for these treatments. Animals were euthanized when they reached the humane endpoint.

5.2.3 Animal Procedures

Most of the procedures are the same of the ones described in **Chapter 4**, such as for animal care and monitoring, X-rays, euthanasia, and histology. Particular care was taking in monitoring the animal's weight, since we have previously seen that the "aged" animal had feeding difficulties. Slight modifications were made to the histological grading system of the skin. In fact, it was noted that the grade assigned to skin and adnexa of the same section varied between two experts. This difference was due especially to discrepancies between the grade assigned to the adipose tissue and to the skin/adnexa conditions. Therefore, it was decided to implement the grading system considering separately the adipose tissue and the skin/adnexa, as shown in **Tables 5.1** and **5.2**. Then the scores described in **Table 5.1** and **5.2** were added to each other and the final condition (**Table 5.3**) was considered for skin final grading and statistical analysis.

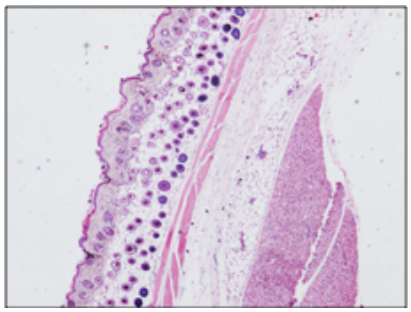
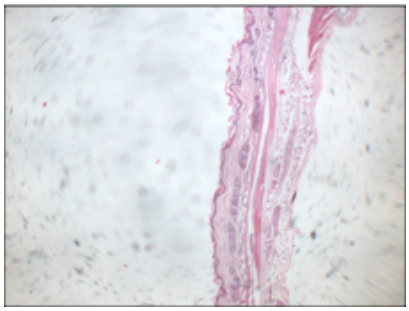
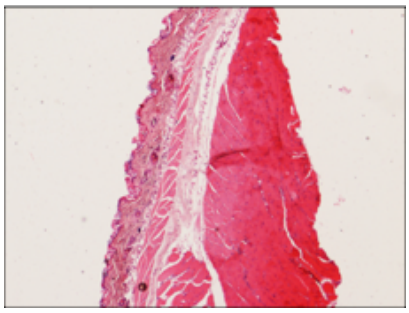
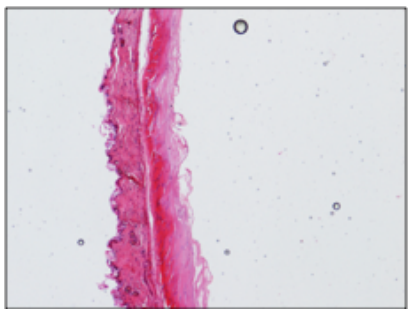
Skin alteration - description	Score	Image
Normal - Normal skin with numerous hair follicles having their bulb in the adipose tissue	**0**	
Mild - Mild reduction of hair follicles that start to lose their contact with subcutis	**1**	
Moderate - Moderate to severe reduction of hair follicles	**2**	
Severe - Rare hair follicle	**3**	

***Table 5.1**: Skin grading system. Grades normal, mild, moderate and severe are described and associated to their score. Skin sections EE, 4X as examples.*

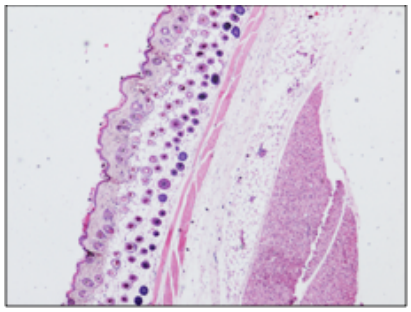
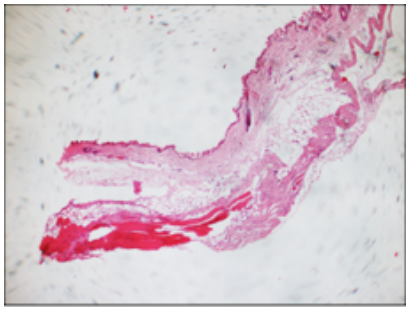
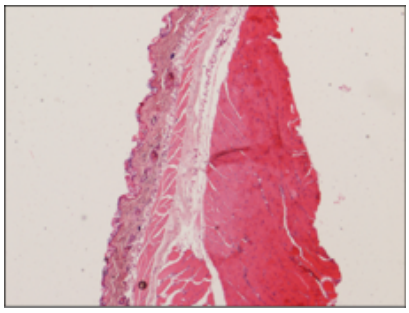
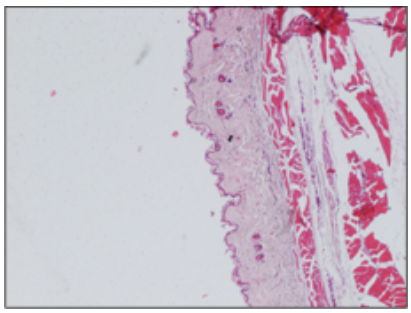
Adipose tissue - description	Score	Image
Normal - Normal abundant adipose tissue	**0**	
Mild - Normal abundant adipose tissue and multifocal reduction	**1**	
Moderate - Small amount of adipose tissue	**2**	
Severe - Atrophy of adipose tissue	**3**	

***Table 5.2**: Adipose tissue grading system. Grades normal, mild, moderate and severe are described and associated to their score. Skin sections EE, 4X as examples.*

Grade	Final skin score
Normal	0-1
Mild	2
Moderate	3-4
Severe	5-6

***Table 5.3**: Grading system for skin. Final scores derive from the sum of the skin/adnexa (Table 5.2) and the adipose tissue (Table 5.3).*

5.2.4 Statistical Analysis

We performed statistical analysis of the differences between treated and untreated mice with a two-tailed Student's *t* test. In experiments with more than two groups (KI), differences were analyzed by multifactorial one-way analysis of variance (ANOVA). For lifespan comparison of different groups in Kaplan-Meier survival plots, a log-rank (Mantel-Cox) test was used. Graphpad Prism 7 software for Macintosh was used for calculations and results are expressed as means±SEM or medians.

5.3 Results

Study 1. *Lmna$^{G609G/+}$*

Five *Lmna$^{G609G/+}$* mice were treated with 1 mg/kg of rapamycin and 0.4 mg/kg of all-trans retinoic acid, twice weekly. Results were compared with untreated mice.

No animals showed side effects imputable to the treatment. The median survival of treated and untreated mice was 292 and 291, respectively. Maximum survival was 368 days for treated mice and 313 for untreated mice. However, the difference in lifespan among groups was not significant ($p > 0.05$) (**Fig.5.2**).

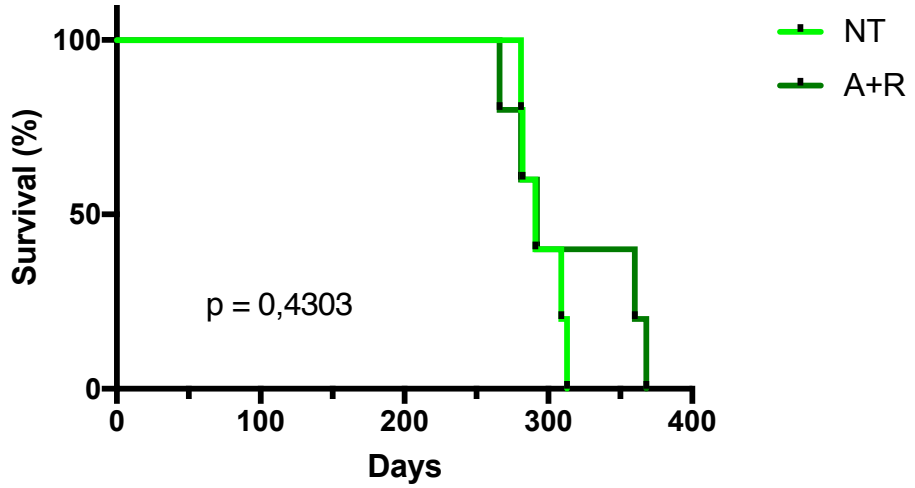


***Fig. 5.2**: Kaplan-Meier survival plots for treated (A+R) and untreated (NT) Lmna$^{G609G/+}$ mice.*

Weights during life of both groups did not vary significantly.

During the open field test (OFT) the two groups of animals acted the same way, crossing the same number of quadrants and doing the same quantity of vertical movements. They also reached the same scores with the numerical scoring system (NSS) described in **Paragraph 4.2.5** used to grade the health status of *Lmna^{G609G}* transgenic mice. When comparing skin scores, aorta scores and kyphosis index among groups no differences were significant ($p > 0.05$) (**Fig. 5.3**). However, we must say, that the first aim of the treatment was to increase the lifespan of the animals and that all the animals considered in this study were sacrificed because they had reached the humane endpoint. This, did not make it possible to evaluate histological improvements during the life of the animals at scheduled times, which should be considered in future studies.

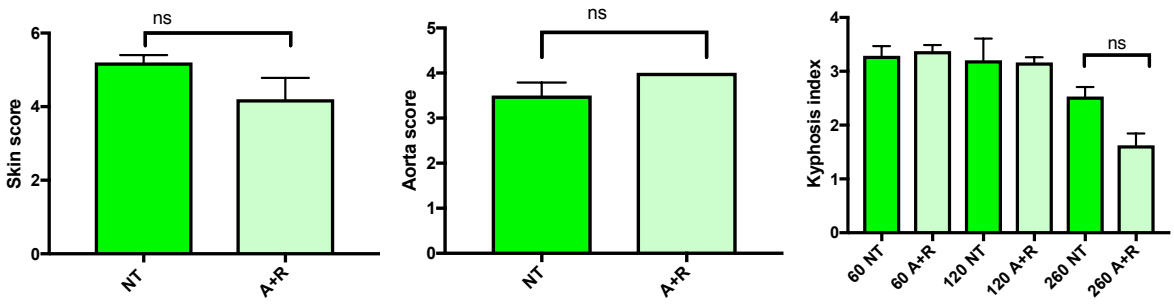


***Fig. 5.3**: Comparison between treated (A+R) and untreated (NT) Lmna$^{G609G/+}$ mice for skin score, aorta score and kyphosis index. Ns= not significant (p > 0.05).*

Lifespan is affected by many factors, and longer the life is more impact these factors might have on it. High variability in lifespan in heterozygous groups may reflect such influence. Furthermore, a life-long therapy administrated IP in mice can accumulate many errors, adding variability to the results.

Doses were chosen based on *in vitro* testing that showed improvements in the amount of progerin in fibroblast from HGPS patients treated with low doses of rapamycin and all-trans retinoic acid. We speculate that the low dosage in our study, was not sufficient in reducing progerin *in vivo*. Thus, dosages might have to be increased in order to detect some effects on the animal model of HGPS.

Study 2. A+R in *Lmna*$^{G609G/G609G}$

This Study has the same aim of Study 1 with the only difference that *Lmna*$^{G609G/G609G}$ mice were used. Some researches believe that such genotype is the best animal model available for preclinical testing of both therapeutic approaches to HGPS and age-related pathologies derived from accumulation of progerin. Therefore, 5 mice were treated with 1 mg/kg of rapamycin and 0.4 mg/kg of all-trans retinoic acid, twice weekly. Results were compared with untreated mice.

Median survival of A+R treated and untreated mice were 115 and 114, respectively. Maximum survival was 128 days for treated mice and 121 for untreated mice. No significant differences ($p > 0.05$) were evidenced in the two groups survivals (**Fig. 5.4**) and body weights.

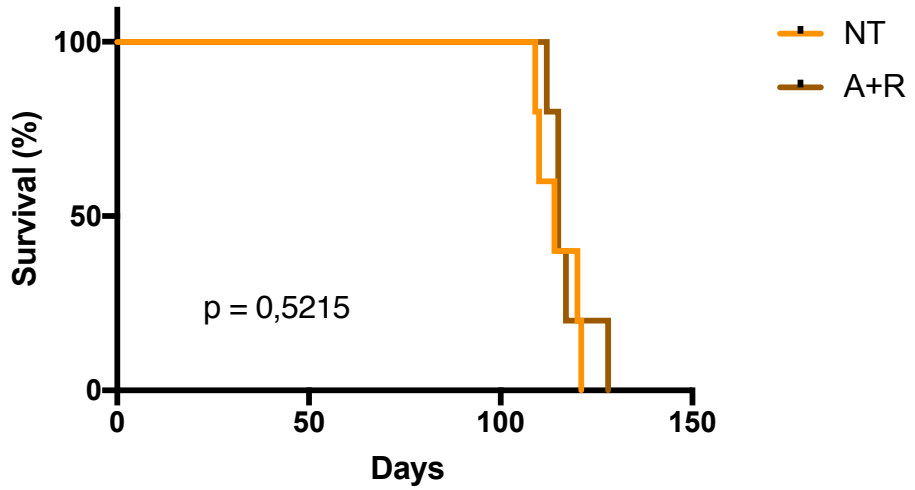


***Fig. 5.4**: Kaplan-Meier survival plots for treated and untreated Lmna*$^{G609G/G609G}$ *mice.*

When comparing skin scores, aorta scores and kyphosis index among groups no differences were significant (**Fig. 5.5**).

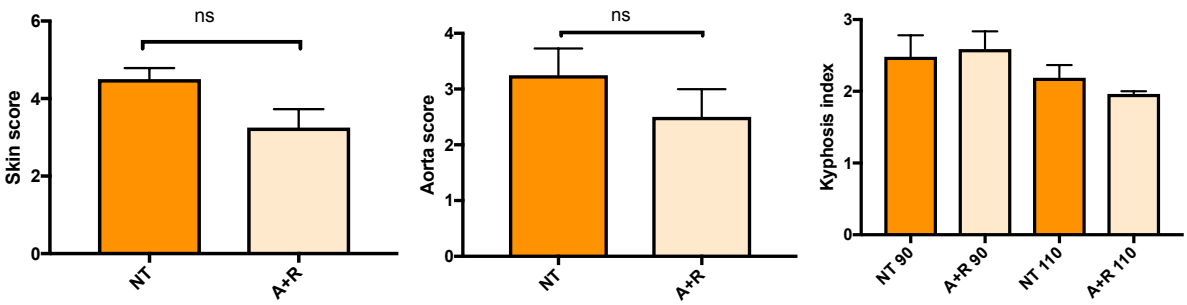


***Fig. 5.5**: Comparison between treated (A+R) and untreated (NT) Lmna*$^{G609G/G609G}$ *mice for skin score, aorta score and kyphosis index. Ns= not significant (p > 0.05).*

This study was performed in homozygous male mice that have an average lifespan of only 115 days (**Chapter 4**), and treatments were started around 50 days of age, when reduced weight among other symptoms are already evident. Starting treatment earlier might have a favorable outcome. However, like in Study 1, we suggest that higher doses are needed in order to make ATRA+RAPA treatment effective.

5.4 Discussions and Conclusions

Following the approval of the experimental protocol (July 2016), preclinical studies were started in order to improve pathological features of the *Lmna*^{G609G} transgenic mice and prolong their lifespan. In this regard, we tested a combination therapy of all-trans retinoic acid and rapamycin. Human HGPS patients carry the *Lmna* mutation in heterozygosis, however some researches think that the progeroid mice carrying the same mutation in homozygosis (*Lmna*$^{G609G/G609G}$) might be a better animal model than the heterozygous since mice evidenced higher tolerance to humans when it comes to accumulation of prelamin A forms (Osorio et al., 2011). As discussed in **Chapter 4**, both models have their pros and cons. Therefore, we conducted this study using both genotypes. Furthermore, we compared data from the same sex groups since differences in lifespan resulted significantly different between males and females. In several published preclinical studies (Osorio et al., 2011; Osorio et al., 2012; Lee et al., 2013) sex of the treated animals was not specified. Some of the traits examined in those studies, such as bone density and resistance, lifespan and weight trends could be influenced by sex-linked biological factors and it is not possible to asses if treatment impacts to a greater extend males or females. Villa-Bellosta et al. (2013) when evaluating pyrophosphate treatment using only males *Lmna*$^{G609G/G609G}$, did not observe differences in body weight and mortality. However, the post-mortem analysis demonstrated a statistically significant reduction in aortic calcification of treated mice compared to the untreated, which was the treatment target.

The IP injection is made through the abdominal wall into the peritoneal cavity. Substances diffuse across the peritoneal membrane which is a semipermeable membrane, lined with a capillary bed. The blood vessels supplying and draining the abdominal viscera, musculature and mesentery, constitute a blood-filled compartment into which drugs can diffuse from the peritoneum. However, there is no visual confirmation that the injection has been correctly administered (as there is, for example, with the intravenous or intradermal routes). For this reason, correct technique is particularly important (Das & North, 2007). One of the major consequences of IP injection failure may be a substantial increase in the apparent variability of the measured responses (Das & North, 2007). Some form of 'partial' administration of the IP injection may also contribute to the variability of the responses (Das & North, 2007). If properly done as described in **Paragraph 5.2.2**, IP injection is easy and efficient and usually achieves successful outcomes of drug research due to accurate, reliable, convenient dosing

with reproducible results because of the large absorbing surface area at the site of injection. Furthermore, compared to other available routes IP causes less distress, assures full administration of the drug and prevents environmental degradation (which could happen when administering through beverage or feed).

Setting up of therapeutic trials for rare diseases, such as HGPS, is very challenging because of the lack of extensive clinical longitudinal studies with homogeneous evaluation parameters on cohorts of patients, which hinders the definition of homogeneous therapeutic outcome measures and endpoints (Osorio et al., 2011). Also, multicentric trials are difficult to organize because of the very low number of patients within a single country, which hampers the strict application of identical protocols in various participating clinical investigation centers (Osorio et al., 2011). Using a well-known animal model that mimics the phenotypic traits of humans helps overcoming such problem.

All-trans retinoic acid (ATRA) is the carboxylic form of vitamin A, and it is known to play a major role in a number of physiologic pathways such as cell proliferation, embryogenesis, differentiation, morphogenesis, and inflammation (Mark et al., 2004). At a dose of 0.5 mg/kg administered IP three times a week for nine weeks it was able, alone, to suppress the clinical and histologic signs of arthritis in a mice model of rheumatoid inflammation (Kwok et al., 2012). Furthermore, ATRA treatment was able to reduce the amount of progerin in HGPS cells and was more effective than rapamycin treatment alone (Pellegrini et al., 2015). ATRA efficacy in reducing progerin was elicited by the combination with low doses of rapamycin (Pellegrini et al., 2015).

Rapamycin is an inhibitor of mTOR kinase that has been demonstrated to increase animal's (from worms to mice) lifespan in a number of studies. Rapamycin is thought to operate as a gerosuppressant, meaning that it inhibits the cellular conversion to a senescent state (the so-called geroconversion), a fundamental process involved in aging and age-related pathologies including cancer (Blagosklonny, 2014). For example, 4 mg/kg IP every other day for six weeks in old C57BL/6 increased mice lifespan (Chen et al., 2009). Anisimov et al. (2010, 2011) reported that FVB/N HER2/*neu* mice and 129/SV female mice treated with 1.5 mg/kg SC three times a week for two weeks and 2 weeks each month, respectively, had an increased lifespan and the treatment prevented age related weight gain. Such treatment was still effective in prolonging lifespan in FVB/N HER2/*neu* when the dose was decreast at 0.45 mg/kg SC (Popovich et al., 2014), starting at 4 and 5 months of age. However, a lack of effect of

rapamycin on the mean lifespan was reported when starting treatment at 2 months of age, and this result was explained by some deaths in mice during early age and thought to be accidental or related to the effects of rapamycin at very young age (Popovich et al., 2014). When administered encapsulated in feed, rapamycin increased median and maximal lifespan in both females and males mice (Harrison et al., 2009; Miller et al., 2014). In particular, rapamycin was demonstrated to increase lifespan more in females than in males (Miller et al., 2014), and this result was correlated to lower levels of rapamycin in females' blood. Although blood levels of rapamycin could have been related to feed consumption, it was concluded that females are likely to have higher blood levels of rapamycin than males, at any age, given equal doses of the drug (Miller et al., 2014). Selman et al. (2009) reported that lifespan was increased in females, but not in males, of Sirtuin 6 kinase 1 knock out mice with a C57BL/6 background. Again, Lamming et al. (2012) demonstrated that inactivation of mTORC1 increased lifespan in females but not in males. The mechanisms through which rapamycin prolongs lifespan are still uncertain. Even though rapamycin was thought to be effective because it causes tumour suppression, some research groups separated the anti-cancer and anti-aging activity of the drug (Anisimov et al., 2011; Wilkinson et al., 2012; Kondratov & Kondratova, 2014). Rapamycin, as any other drug, has side effects in humans mostly related to high doses (Blagosklonny, 2014; Kondratov & Kondratova, 2014). Also, life-long chronic exposure to rapamycin, while preventing most age-related diseases and extending healthspan, was shown to increase incidence of cataracts and some other alterations in mice (Wilkinson et al., 2012). However, the ability of rapamycin to work at low doses makes it substantially more attractive as a candidate for a preventive medicine (Kondratov & Kondratova, 2014). Rapamycin, acting as a mTOR inhibitor, was shown to contribute to progerin degradation by activating autophagy (Bjedov & Partridge, 2011), and *in vitro* studies reported promising results in its use as a therapeutic approach to Progeria syndrome (Cao et al., 2011; Cenni et al., 2011; Mendelsohn & Larrick, 2011), especially when used at low doses in combination with ATRA (Pellegrini et al., 2015). In April 2016, a phase I clinical trial started (and is still ongoing) to determine the maximum tolerated dose of everolimus (rapalog) that is taken in combination with Lonafarnib in human patients with HGPS (PRF, 2017c).

In this preliminary study, a combination of low dose rapamycin (1 mg/kg) and all-trans retinoic acid (0.4 mg/kg), administered during the whole animal's life twice weekly starting from 6 weeks of age were tested in *Lmna*$^{G609G/+}$ and *Lmna*$^{G609G/G609G}$ transgenic male mice.

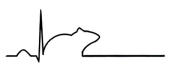
Differences in survival analysis of treated and untreated groups did not reveal significant differences for both genotypes. However, maximal survival was 55 days longer for treated heterozygous compared to untreated, and 7 days longer for treated homozygous. Increasing the number of animal for group might lead to significant differences. Moreover, dose related drug sensitivity could differ among sexes; therefore, it might be interesting to evaluate the effects of such treatment in females who might be more influenced by the treatment. We also did not observe changes in mean weights between groups. It must be noted that ATRA is known to induce body fat loss by activating brown adipose tissue, reducing lipogenic capabilities and increasing oxidative metabolism and thermogenesis in white adipose tissue depots and skeletal muscle (Amengual et al., 2010). Also, treatment with rapamycin is reported to prevent weight gain related with aging (Anisimov et al., 2010; Anisimov et al., 2011). However, we did not register weight loss of the treated group compared to untreated group likely indicating that this does not occur for low doses of ATRA+RAPA administered long-term in such mouse model.

Treated mice did not exhibit changes in the open field test, differently from rats treated with 1 and 3 mg/kg of rapamycin (Lu et al., 2015).

Questions about safe dosage of ATRA+RAPA administered chronically, timing in starting the therapy still need to be answered and are extremely important. In fact, a study demonstrated that rapamycin was successful in prolonging lifespan when administered starting from 4 months of age but not at 2 months (Popovich et al., 2014).

Other studies are needed to investigate weather treatments with higher dosages of the tested therapies, starting at different time points, and the combination of such therapy with farnesyl-transferase inhibitors and statins could be more beneficial than current strategies.

Thanks to the collaboration with the Institute of Molecular Genetics CNR, Bologna, studies aiming to prolong lifespan and increase life quality of *Lmna*^{G609G} transgenic mice are still ongoing.



Chapter 6

Animal Models of HER2-positive Cancers, and Oncolytic Virus-Mediated Immunotherapy

6.1 Introduction

Topics dealt with in this chapter are needed in order to better understand the experimental study discussed in **Chapter 7**. In particular, these topics include HER2-positive cancers and treatments with targeted drugs, oncolytic virus-mediated immunotherapy and animal models for preclinical testing of HER2-positive cancer treatment. A brief discussion on difficulties encountered when translating preclinical results to clinical testing is also held.

6.2 HER2

The year 2017 marks exactly three decades since the Human Epidermal Growth Factor Receptor 2 (HER2) was functionally implicated in the pathogenesis of human breast cancer (Slamon et al., 1987). This finding established the HER2 oncogene hypothesis for the development of some human cancers (Moasser, 2007).

While HER2 is used in reference to the human gene and protein, c-erbB-2 is used to refer to the gene across humans and rodents, and *neu* for the rodent counterparts (Moasser, 2007).

Neu was initially described as a transforming oncogene in a carcinogen induced rat brain tumour model (Shih et al., 1981). Four years later, in an independent study, an EGFR-related gene was found to be amplified in a human breast cancer cell line and was named Human Epidermal Growth Factor Receptor 2 (King et al., 1985). Subsequent cloning of two other related human genes and the post-genome characterization of the human kinome completed the description of this family of four members (Kraus et al., 1989; Plowman et al., 1993; Manning et al., 2002; Moasser, 2007), therefore composed by EGFR (HER1, erbB1), HER2 (erbB2, HER2/*neu*), HER3 (erbB3), and HER4 (erbB4).

The HER2 is a proto-oncogene encoding a 185 kDa transmembrane glycoprotein, as well named HER2, described in different tumours and animals (de las Mulas et al., 2003; Ma et al., 2013). Such glycoprotein is a tyrosine kinase receptor that has a similar structure in all HER family members. The structure is made of a glycosylated extracellular domain (ECD), an hydrophobic transmembrane domain, and an intracellular domain with tyrosine kinase activity. When specific ligands bind to the ECD they cause a conformation change that leads to dimerization. Such dimerization induces tyrosine kinase phosphorylation and downstream signalling causing cell migration and proliferation, as well as cell survival (Barthelemy et al., 2014). However, this is not true for HER2 for which no specific ligands have been identified and is constitutively present at the cell surface in an active conformation (**Fig. 6.1**). Therefore, it is considered an optimal partner for dimerization of other HER proteins and it has been the goal of novel targeted cancer therapies.

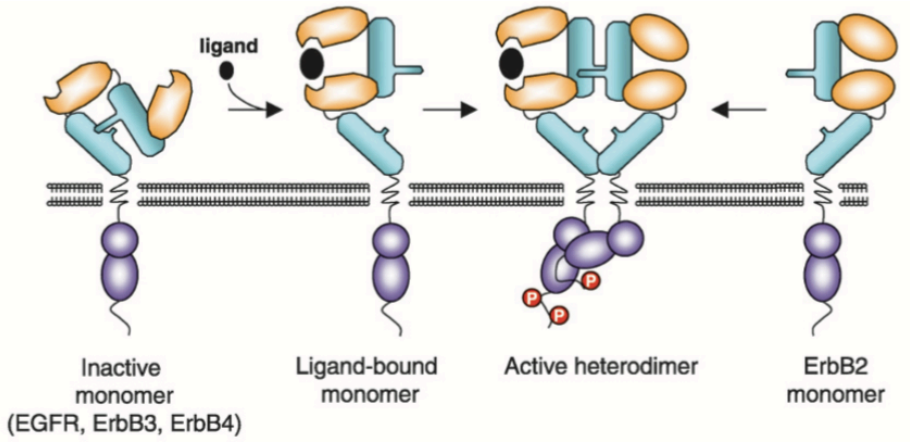


***Fig.6.1**: Schematic overview of the structural basis for HER receptor dimerization and activation.*
HER1, HER2 and HER3 have a closed conformation. When the ligand binds to them it creates an extended conformation, allowing for receptor homo- and heterodimerization. The dimerization leads to the C-terminal tyrosine phosphorylation, creating phosphotyrosine binding sites for binding of adaptors, signalling molecules and regulatory proteins. HER2 is fixed in the active conformation and therefore ready to interact with other HER receptors (adapted with permission from Wieduwilt & Moasser, 2008).

Significant differences were thought to be present between human and rodent genes: while human HER2 appears to hold tumorigenic potential through overexpression alone, rodent *neu* appears to require mutational activation (Moasser, 2007). However, recently rare types of human lung cancers that have HER2 kinase domain mutations, conferring increased kinase activity without overexpression, might be in contrast with previous observations. To date, to little is known on such domain to challenge the overexpression model (Moasser, 2007).
What is a fact, it that overexpression of HER2 correlates in humans with more aggressive clinic pathologic features, drug resistance or sensitivity to specific chemotherapy and specific hormonal therapy regimens in breast cancer (Slamon et al., 1987; Revillion et al., 1998; Sahin, 2000). In node-positive breast cancer it may be associated with worse outcomes even at low-level expression (Gilcrease et al., 2009). Apart from breast cancer, HER2 overexpression and amplification has been detected in many other human tumours, including gastric, oesophageal, endometrial, ovarian, salivary gland, oropharynx, bladder, pancreatic, lung and melanoma cancers (Hirsch et al. 2002; Khan et al. 2002; Latif et al. 2003; Glisson et al., 2004; Hansel et al., 2005; Mimura et al. 2005; Morrison et al., 2006; Yano et al., 2006; Marín et al., 2010; Ma et al., 2013; Pagni et al., 2013). Numerous transgenic mouse models have validated the theory that HER2 has an important role in tumorigenesis.

6.3 HER2 Diagnosis

As it can be seen from the following paragraph, the development of anti-HER2 therapeutic agents represents a great success in individualized therapy, especially for what concerns HER2-positive breast cancers. Therefore, in a standard day of routine diagnostic practice HER2 scoring, along with the assessment of other prognostic and predictive factors, is undoubtedly one of the topic moments in terms of breast cancer pathology (Sapino et al., 2013).

Obviously, it is mandatory to have a proper sampling and biopsies need to account for tissue heterogeneity. From a laboratory point of view, different methodological approaches are available.

The first step is the assessment of HER2 protein overexpression in immunohistochemistry (IHC) (Marques et al., 2016). A four-tier scoring system is contemplated in which, depending on the final score, further tests could be needed in order to confirm eligibility to treatment (Wolff et al., 2007; Sapino et al., 2013). It has been demonstrated that there is a number of false negative/false positive depending on the use of the scoring system (Perez et al., 2012; Wolff et al., 2012), so IHC is not considered to be robust (Sapino et al., 2013).

HER2 gene amplification is present in 85–90% of the cases (Ratcliffe et al., 1997; Isola et al., 1999; Hoang et al., 2000; Jimenez et al., 2000; Johnson et al., 2000), and three main methods are approved to detect it. In particular:

- fluorescence *in situ* hybridization (FISH)
- chromogenic *in situ* hybridization (CISH)
- silver *in situ* hybridization (SISH).

FISH was considered for many years to be the gold standard method for detecting HER2 amplification, but is not very practical for routine histopathological laboratories. Thanks to the development of a “fast FISH” (IQFISH) with a 98% concordance with traditional FISH (Matthiesen & Hansen, 2012), the turnaround time output has been shortened.

There are many issues on reproducibility and reliability of HER2 testing and the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) published a special article that serves as a guideline for HER2 testing (Wolff et al., 2014).

6.4 HER2 Targeted Drugs

Targeted cancer therapies interfere with specific molecules that are involved in growth, progression and spread of cancer. Targeted cancer therapies are also sometimes called "molecularly targeted drugs," "molecularly targeted therapies," "precision medicines," or have other similar names (NCI, 2017).

Several drugs have been developed and are in clinical use to block the HER pathway (Patel et al., 2014). In 1998, trastuzumab, a monoclonal antibody directed against HER2, was the first monoclonal antibody and HER-directed therapy approved by the FDA for metastatic breast cancer therapy (Slamon et al., 2001). Trastuzumab is commonly used in combination with a wide range of chemotherapy agents (Patel et al., 2014) as a first line treatment for HER2-positive metastatic breast cancer. It is also approved in combination with chemotherapy, for the treatment of HER2-positive metastatic cancer of the stomach or gastroesophageal junction (Herceptin, 2017). It is thought to cause death of HER2 overexpressing cells through mechanisms involving induction of apopstosis, inhibition of HER2 cells proliferation signalling and antibody-dependent cell-mediated cytotoxicity (Arteaga et al., 2012).

Lapatinib is a HER1/HER2 kinase inhibitor that blocking signal transduction pathways has demonstrated activity in HER2 overexpressing breast cancer and was approved in 2007 as second-line therapy for metastatic breast cancers (Rusnak et al., 2001; NCI, 2011).

Pertuzumab is a recombinant humanized monoclonal antibody approved in combination with chemotherapy and trastuzumab for the first-line treatment of HER2-positive metastatic breast cancer and for the neoadjuvant therapy of HER2-positive (NCI, 2013). It acts by blocking HER2 to dimerize with other HER receptors, such as with HER3, by binding to the extracellular dimerization domain II of HER2 (Patel et al., 2014). Thus, pertuzumab, targets a different epitope from that of trastuzumab, which blocks the signalling pathway without affecting dimerization (Barthelemy et al., 2014).

Ado-trastuzumab-emtansine (T-DM1) is a new class of antibody–drug conjugate that has recently shown superior clinical activity, combining the effect of trastuzumab with a cytotoxic agent. The conjugate is internalized via receptor-mediated endocytosis in HER2 overexpressing cells, and an active derivative of DM1 is subsequently released by proteolytic degradation of the antibody moiety within the lysosome (Lambert & Chari, 2014). Such delivery system improves the therapeutic index and minimizes exposure of normal tissue (Verma et al., 2012; Patel et al., 2014).

Most of HER2 targeted therapies are approved for breast cancer. However, many therapies are being investigated in preclinical and clinical trials for other HER2-positive cancers.

Targeted therapies are currently at the centre of anticancer drug development. Despite the success of single agents targeting the HER family, there are a number of escape mechanisms from HER-targeted therapies suggesting both acquired and *de novo* mechanisms of drug resistance (Patel et al., 2014). A single-agent block might provide an escape mechanism through the redundancy in the input layer of the network (Patel et al., 2014). Many studies have demonstrated that single-agent HER2 targeted therapies are efficacious but that the response is incomplete. Only a subset of HER2-positive cancers responds to current targeted therapy, and resistance is especially driven by alterations in HER2 signalling pathway rather than its surface expression (Menotti et al., 2009). Thus, more effective and innovative therapies are needed against this tumour target.

Oncolytic virotherapy is an emerging treatment modality which uses competent replicant viruses to destroy cancers (Russel et al., 2012). Viruses can be genetically modified to recognize some receptors as targets; in these cases, receptors that are uniquely expressed or overexpressed by tumour cells, such as for HER2 glycoprotein, are chosen. Specific targeting of cancer cells is obviously essential for oncolytic virotherapy (Russel et al., 2012). Often, it is also important to detarget the viruses from their natural receptors (Cattaneo et al., 2008; Gao et al., 2008). A number of oncolytic viruses (OVs) which induced tumour regression in animal models of human cancers are now being assayed in clinical trials (Leoni et al., 2015). Some of these are under trial for HER2-positive cancer treatment (NIH, 2017).

6.5 Oncolytic Virus-Mediated Immunotherapy

As previously mentioned, oncolytic virotherapy uses the ability of viruses to replicate and kill target cells and simultaneously spread to other target cells (Nanni et al., 2013). This concept is not completely new since throughout the twentieth century several mini trials or case reports described the use of crudely prepared clinical or laboratory viral isolates for oncotherapy (Chiocca, 2002). Only in 1991 the first anticancer virus was engineered and used in a preclinical model of human glioma (Martuza et al., 1991). Since then, many viruses have been genetically modified to generate replicating oncolytic agents that target tumour cells with varying extent of cancer specificity (Leoni et al., 2015). Interest in OVs has been increasing based on a better understanding of viral biology, tumour immunology and molecular genetics (Kaufman et al., 2015).

The outcome of viral infections can vary greatly depending on the pathogenic nature of the virally encoded genes, interactions between the virus and the host immune system and the ability of the virus to replicate and/or induce latency following infection (Kaufman et al., 2015). OVs are thought to mediate antitumor activity through two main mechanisms: selective replication inside cancer cells that causes cell lysis, and induction of systemic antitumor immunity (Kaufman et al., 2015). In particular, the immune response to oncolytic viruses is apparently an important component of the antitumor effect (Kaufman et al., 2015). In fact, OV-lysed tumour cells release 1) tumour associated antigens; 2) viral Pathogen-Associated Molecular Patterns (known as PAMPs - such as, capsids, DNA, proteins etc); 3) Danger-Associated Molecular Patterns (known as DAMPs - such as heat shock proteins, ATP, uric acid etc); and cytokins (type 1 IFN, TNFα, IFN γ, IL-12). The release of these components is important for inducing innate and adaptive immune responses against cancer cells. The innate immune response is not antigen specific, does not lead to lasting immune memory and doesn't increase with repeated exposure to the antigen. Key partecipats of innate immunity are macrophages, neutrophils, natural killer (NK) cells, and dendritic cells (DCs). The adaptive immune response starts when DCs ingest an antigen, migrate to the peripheral lymphoid tissue, and mature into antigen-presenting cells. T-lymphocytes are then activated. Especially cytotoxic $CD8^+$ T cells have been demonstrated to be important in mediating tumour rejection because of their ability to reach sites of established tumour growth, where they mediated anti-tumour immunity upon antigen recognition (Kaufman et al., 2015; Aurelian, 2016).

Nonetheless, the co-participation of the immune system could represent a limit since neutralizing antiviral responses, especially mediated by the innate immunity, may block virus replication and ongoing infection of tumour cells (Kaufman et al., 2015; Aurelian, 2016). Such ability of the immune system to neutralize the virus depends on many variables, but especially on the virus characteristics and on the tumour microenvironment (Kaufman et al., 2015). Chronic activation of innate immunity appears to be correlated with poor OVs treatment prognosis (Aurelian, 2016). Clinical efficacy is therefore a delicate balance between these factors, among others.

Oncolytic viruses (OVs) encompass a broad diversity of DNA and RNA viruses that are naturally cancer-selective or can be genetically manipulated in order to gain selectivity (Chiocca & Rabkin, 2014). OVc are based on adenovirus, herpes virus, reoviruses, retroviruses, measles viruses, vaccinia viruses and many other types.

In 2004 in Latvia, a non-genetically engineered oncolytic, non-pathogenic enteric cytopathic human orphan type 7 (ECHO-7) virus adapted and selected for melanoma (Rigvir), was approved for melanoma therapy in humans (Babiker et al., 2017). In 2006, the oncolytic virus H101, a modified adenovirus, was approved in China, for the treatment of squamous cell carcinoma of head and neck (Xia et al., 2004; Babiker et al., 2017). In October 2015, talimogene laherparepvecan (T-VEC, Amgen), an herpes simplex virus type 1–derived OV, was the first FDA approved OV for intratumoral treatment of melanoma. T-VEC is able to replicate selectively in tumour cells and has been engineered to provoke anticancer immunity via expression of granulocyte-macrophages colony-stimulating factor (GM-CSF) (Lawier & Chiocca, 2015). To date, other OVs completed phase I and II clinical trials for glioblastoma, other solid tumours, as well as for leukemia and lymphoma (Aurelian, 2016). It is important to note that all OVs trials haven't so far had serious adverse events, and that the tolerable and safe profiles make combination treatments feasible. Apparently, one of the most promising strategies is to combine OVs with T cell checkpoint inhibitors, which are potent agents with activity in a wide range of cancers. In particular, studies suggest that patients with tumours expressing high levels of programmed cell death 1 ligand 1 (PDL1) may have an improved response to T cell checkpoint inhibitors. As oncolytic viruses often induce INF release in the local tumour microenvironment, and IFN is known to upregulate PDL1 expression on tumour cells, this combination is especially interesting (Kaufman et al., 2015).

OVs safety can be achieved through various mechanisms, most importantly by virus attenuation (Mineta et al., 1995; Hunter et al., 1999). An alternative approach to attenuation has been to design OVs fully retargeted to cancer-specific receptors and detargeted from natural receptors, especially for what concerns herpes viruses (Campadelli-Fiume et al., 2016). Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus, member of alphaherpesvirus family and a major human pathogen that causes skin lesions, rashes and can infect peripheral nerves and enter a latent stage (Kaufman et al., 2015). This virus can infect epithelial cells by viral surface glycoproteins, neurons through surface nectins and the immune cells through the herpesvirus entry mediator (HVEM) (Kaufman et al., 2015). Fortunately, HSV-1 has a large genome (152 kb), in which about 30 kb encode genes that are not essential for viral infection. This means that there is space to insert heterologous sequences and also that it can be relatively easy to genetically manipulate and to detarget it from its natural receptors. Furthermore, in the worst-case scenario, there is the possibility to control any unwanted replication in humans by acyclovir treatment (Menotti et al., 2008; Leoni et al., 2015). For these reasons among others, HSV-1 represents a highly promising OV.

OV antitumor activity can be enhanced by transgene arming, including monoclonal antibodies, proapoptotic genes, antiangiogenic and antivascular proteins, enzymes that degrade extracellular matrix and inflammatory cytokines (Aurelian, 2016). As previously said, for example, T-VEC expresses the immunostimulatory cytokine GM-CSF. Arming OVs with immune-enhancing cytokines capable of boosting the host's immune response to effectively attack tumour cells appears promising. In particular, a number of OVs that harbour IL-12 has been demonstrated to have an enhanced anticancer immune response. IL-12 is one of the most important anticancer cytokines, and its effect is mediated by the activation and recruitment of DCs, cytotoxic NK and cytotoxic T cells and by an antiangiogenic effect (Alkayyal et al., 2016).

Special challenges in OVs development remain the need for more practical clinical trials designs, validated PK/PD assays, biosafety issues and non-traditional regulatory, manufacturing and commercialization issues (Kaufman et al., 2015).

6.6 HER2 Animal Models and Translational Relevance in Veterinary Medicine

Most of the animal models for HER2 have been used to demonstrate the histopathology, oncogenic signalling pathways initiated by aberrant overexpression of HER2 in cancers, and oncogenes-tumour suppressor genes at molecular levels interactions (Fry et al., 2016).

For example, canine mammary gland carcinomas have epidemiological, clinical, morphologic and prognostic features similar to those of human breast carcinoma, for which they are thought to be suitable natural models (de las Mulas et al., 2003; Frese, 1986; Nerurkar et al., 1989; Withrow & MacEwn, 2001). In particular, a HER2 homologue with 92% amino acid identity has been described in canine mammary tumours, known as "dog epidermal growth factor receptor 2 (DER2)", with similar biological implications as those in human breast cancer (Singer et al., 2012; Fazekas et al., 2016). Also, the percentage of HER2 protein overexpression among mammary carcinomas in humans and dogs are similar (de las Mulas et al., 2003). However, no amplification of the c-erbB-2 oncogene was detected in the canine tumours, while the human species, depending on the diagnostic techniques, show oncogene amplification in 85–90% of the cases overexpressing the protein (Ratcliffe et al., 1997; Isola et al., 1999; Hoang et al., 2000; Jimenez et al., 2000; Johnson et al., 2000). Thus, canine mammary carcinomas would be suitable natural models of that subset of human breast carcinomas with HER2 protein overexpression without gene amplification (de las Mulas et al., 2003), especially considering the high incidence of such cancer in dogs. In fact, incidence is estimated to be at 50%, of which 40 to 50% are diagnosed as malignant (Baba & Catoi, 2007). More importantly, it is clear that dogs with naturally occurring HER2-positive cancer would not only act as animal model patients for human disease, but would gain individual benefit by participating in a clinical trial (Paoloni & Khanna, 2008; Queiroga et al., 2011) (**Fig. 6.2**).

Highly variable levels of HER2 expression have been also recorded in feline mammary tumours. The numbers of positive tumours range from 5.5% to 90% in different studies, probably due to antibody selection, which is the criteria for assessing expression, among other issues (de Maria et al., 2005; Millanta et al., 2005; Winston et al., 2005; Ordas et al., 2007; Rasotto et al., 2011; Hughes & Dobson, 2012). Some authors have demonstrated a correlation between HER2 expression and overall survival (Millanta et al., 2005), however a recent study indicated that HER2 might have less prognostic potential in feline mammary tumours than woman breast cancer (Rasotto et al., 2011). As for dogs, reports showed low HER2 mRNA levels and no gene amplification in HER2-positive feline mammary carcinoma (Ordas et al.,

2007; Soares et al., 2013). If on one side further research is needed in order to prove if the feline mammary carcinoma can be a model for human breast cancer, on the other hand the high prevalence of HER2 overexpression can be a great opportunity to study specific targeted drugs (Marques et al., 2016).

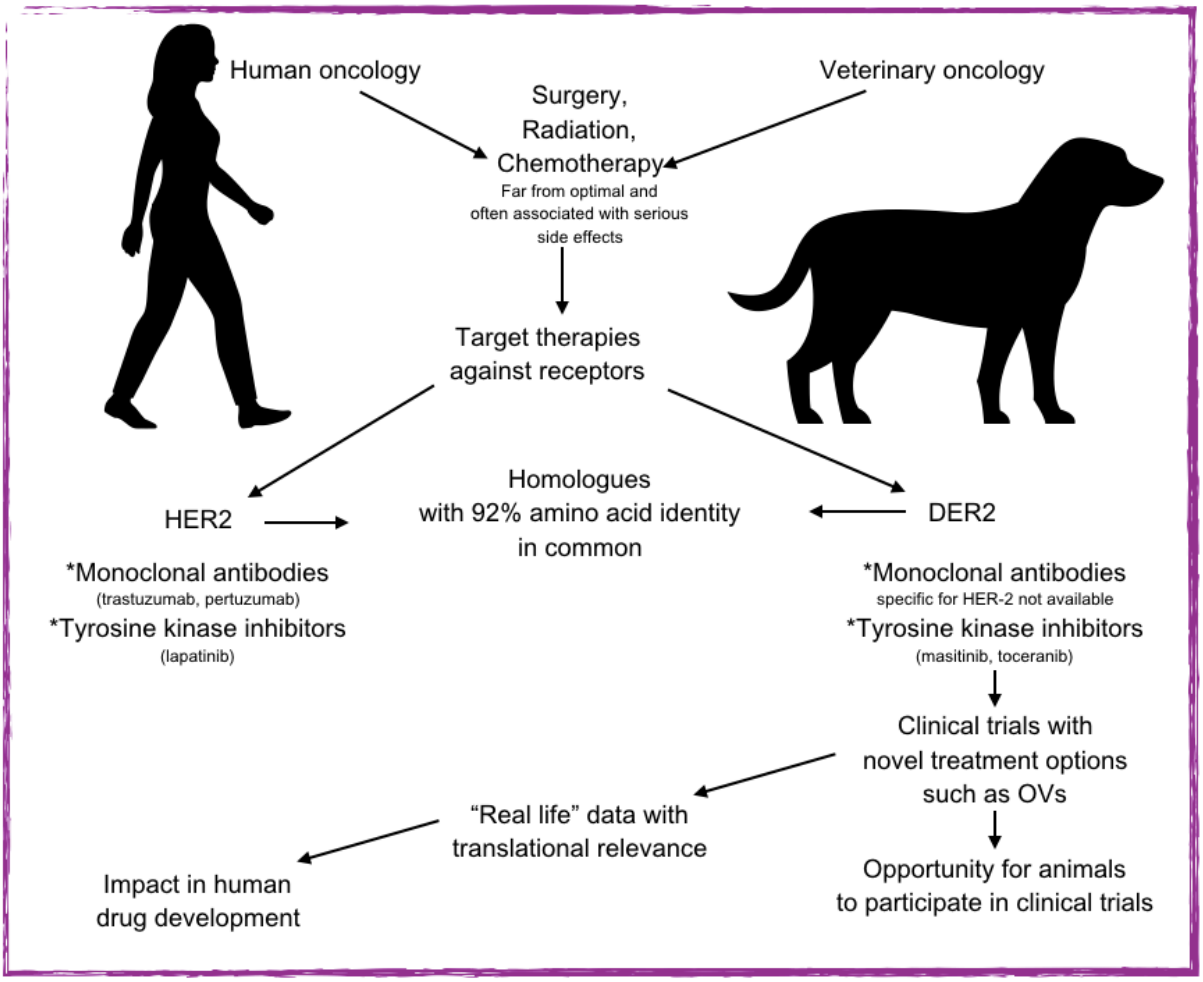


***Fig. 6.2**: How clinical trials in canine cancer patients could contribute to the development of novel precision medicine options in human and veterinary oncology.*

Again, animal patients with similar spontaneous diseases to humans are usually left out during the drug development process, even though they could serve as "real life" models for human diseases and are a missing link between the laboratory setting of animal experimentation and the "real life" conditions (Fazekas et al., 2016). Studying pets such as cats and dogs with naturally developing cancers is likely to provide a valuable perspective that is distinct from that generated by the study of human or rodent cancers alone (Paoloni & Khanna, 2008).

In the animal experimentation setting, especially mice have been used as models and have been crucial to increase knowledge about oncogenic mechanisms. However, these models can also be used in preclinical studies to test the efficacy of HER2 targeted therapies.

Since 1980's a number of transgenic mice have been developed, mostly to study human breast cancer. The first transgenic model investigating the role of HER2 in carcinogenesis was in 1988, when Muller et al. (1988) used the mouse mammary tumour virus (MMTV) to activate the *c-neu* (activated *c-neu* referred as *neu-NT*) gene providing transgenic mice that spontaneously developed mammary tumours in a short latency time (Muller et al., 1988). Since then, as it can be seen from **Table 6.1**, MMTV-*neu*-NT, both on FVB or balb/c backgrounds, have been useful not only to study oncogenic mechanisms but also to study a number of targeted therapies and vaccines. Although MMTV-*neu*-NT models among others (briefly described in **Table 6.1**) expressing the *neu* gene are interesting and useful, there is about 10% difference between rat and human erbB-2 proteins (Yamamoto et al., 1986). When immunized with human erbB-2 DNA even though females developed less tumours, both humoral and cellular immunity were not detected and whether tolerance to rat *neu* was overcome remained uncertain (Pupa et al., 2001). Therefore, *neu*-transgenic models might not be suitable for investigating the efficacy of therapeutics targeted to the human HER2. After some failed attempts (Stocklin et al., 1993), a transgenic mouse tolerant for HER2 syngeneic to C57BL/6 background was established (Piechocki et al., 2003). Such HER2 transgenic mouse was achieved using the whey acidic protein (WAP) promoter (in light blue in **Table 6.1**). HER2 mice were tolerant to human HER2 and permissive to the out-growth of tumours expressing HER2 without generating an antibody response, and did not show spontaneous tumour growth (Piechocki et al., 2003). This model is considered to be valuable to test vaccinations and the activity of oncolytic viruses and immunotherapy against different tumour types expressing HER2. One year after this model, Finkle et al. (2004) developed another mouse model of human HER2-overexpressing breast cancer under the control of the MMTV promoter.

Most of HER2/*neu* transgenic models have been achieved by transgene integration using MMTV promoter, with the exception of Piechocki et al. (2003) who used whey acidic protein (WAP) and achieved a model that doesn't develop spontaneous cancers. The animal models used in experimental designs should be chosen on the basis of the aim of the study.

Overall, it is important to recognise both the strengths and the weaknesses of engineered models and models of naturally cancer development, such as dogs and cats. They may be appropriately used and integrated into a comprehensive drug-development programme.
It has been recommended that therapeutic agents, especially in oncology, should not only be evaluated in rodents, but also in higher animal species (Mak et al., 2014).

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-*neu*-NT (in mouse lineage TG.NF)	Early, multiple mammary tumours	3 months of age, short survival	First transgenic model created using the mouse mammary tumour virus (MMTV) to activate the c-*neu*	Results suggested that activated *neu* overexpression could drive mammary carcinogenesis in a single step	Muller et al., 1988
MMTV-*neu*-NT	Mammary tumours stochastically	5-10 months		Longer tumour latency provided evidence that carcinogenesis could depend on the site of integration of the transgene and on the transgene copy number	Bouchard et al., 1989
MMTV-*neu*-NT			Three generation description of the animal model	*Neu* oncogene can induce tumours in all the tissues where it is expressed at high levels	Lucchini et al., 1992
MMTV-*neu*-NT on FVB background (wild type)	Focal mammary tumour after long latency	40 weeks of age	Establishment of a transgenic mice on FVB background	Over expression of *neu* in the mammary tumours was associate with elevated *neu* intrinsic tyrosine kinase activity and the de novo tyrosine phosphorylation of several cellular proteins. Many mice developed secondary metastatic tumours in the lung after long latency	Guy et al., 1992
MMTV-erbB2 (human variant)	Histopathological analysis suggests that preneoplastic lesions in kidney and lung most likely caused organ failure and the early death of the transgenic mice	Death at 4 months	To study the pathogenicity of the human c-erbB-2 oncogene was evaluated in transgenic mice	Description of the model	Stocklin et al., 1993
MMTV-*neu*-NT on Balb/c and FVB background	Invasive carcinomas of all 10 mammary glands	33 weeks of age for balb/c; 48-49 for FVB	Ability of IL-12 in preventing tumours	Tumour inhibition was associated with: mammary infiltration of reactive cells; production of cytokines; inducible nitric oxide synthase; reduction in microvessel number	Boggio et al., 1998

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
K14-erbB-2	Severe hyperplastic lesions of skin, hair follicles, oesophagus, perinatal lethal		To investigate erbB-2 role in the skin	Skin is sensitive to erbB-2 signalling, suggesting an important role for this receptor in epidermal growth, differentiation and hair follicle morphogenesis	Xie et al., 1998
K5.*neu*	Severe hyperplastic lesions of skin, oesophagus, papillomas, early death		To investigate erbB-2 role in the skin	ErbB2 signalling has an important role in epidermal proliferation and carcinogenesis	Bol et al., 1998
MMTV-*neu*-NDL *neu* deletion			To assess the importance of *neu* activation during mammary tumorigenesis, altered receptors harboring in-frame deletions within the extracellular domain were expressed in transgenic mice	Co-expression of erbB-2 and erbB-3 may play a critical role in the induction of human breast tumours, and raise the possibility that activating mutations in the erbB-2 receptor may also contribute to this process	Siegel et al., 1999
K14-rtTA/TetRE-ErbB2 'Tet-On'	Rapid doxycycline induction of hyperplastic lesions in skin, hair follicles, oesophagus, regress without doxycycline		To investigate conditional expression of the erbB2 oncogene in skin	ErbB2 plays important roles in both development and maintenance of hair follicles and diverse squamous epithelia. The model may be useful in studying transgenes with perinatal toxicity	Xie et al., 1999
MMTV-*neu*-NT on Balb/c and FVB background			Ability of systemic IL-12 to hamper progressive stages of mammary carcinogenesis	IL-12 interfered with progression of early preneoplastic lesions while later treatments where less effective	Boggio et al., 2000

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-Cre	Late mammary tumours	12-17 months	To generate transgenic mice that carry the activated *neu* oncogene under the transcriptional control of the endogenous *neu* promoter	Mammary tumorigenesis in *neu* mouse models require the amplification and commensurate elevated expression of the *neu* gene	Andrechek et al., 2000
NN60, TNT	Abnormal involution, focal mammary abnormalities in multiparous females	Between 1 and 2 years	To generate two breast cancer mouse models: one expressing rat wild type *neu*, the other expressing rat mutant *neu,* both under the control of the normal mouse *neu* promoter	Minor perturbations in amplified *neu* expression are sufficient to alter mammary development and induce malignant transformation	Weinstein et al., 2000
BK5.erbB2	Spontaneous papillomas, some of which converted to squamous cell carcinomas	Starting from 6 weeks of age. Survive 6-12 month	To investigate the constitutive expression of erbB2 in epidermis of transgenic mice	The important role of erbB2 signalling in epidermal growth, development and neoplasia	Kiguchi et al., 2000
MMTV-*neu*-NT on FVB background			To test a DNA vaccine in preventing mammary tumour development	The xenogeneic HER2 DNA sequence was able to break immune tolerance to rat *neu* in transgenic mice and induced protective immunity, possibly based on different mechanisms including aspecific and inflammatory immunological responses	Pupa et al., 2001
BK5.erbB2	Gallbladder adenocarcinomas	Starting from 3 months of age. Survive 6-12 month	To investigate the constitutive expression of erbB-2 in gallbladder epithelium	The transgenic mouse obtained was proposed as a new animal model for studying biliary tract carcinogenesis	Kiguchi et al., 2001
MMTV-*neu*-NT on Balb/c background			Inhibition of mammary carcinogenesis by systemic interleukin 12 or p185*neu* DNA vaccination	Elicitation of nonspecific and specific immunity could be beneficially used in individuals with a high risk of developing tumours	Di Carlo et al., 2001

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-*neu*-NDL, *neu*-NDL-YB, *neu*-NDL-YD			To investigate the role of the *neu* autophosphorylation sites in transforming its signalling	Grb2 and Shc play important and distinct roles in ErbB-2/*Neu*-induced mammary tumorigenesis and metastasis	Dankort et al., 2001
MMTV-rtTA/TetO-*Neu*NT	Rapid mammary tumours and frequent lung metastasis	6 weeks	The tetracycline regulatory system to conditionally express activated *neu* in the mammary epithelium of transgenic mice was used to determine the impact of tumour progression on the reversibility of *neu*-induced tumorigenesis	*Neu*-initiated tumorigenesis is reversible	Moody et al., 2002
WAP-HER2 C57BL/6	None of the tissues expressing HER2 demonstrated obvious abnormality. Mice were healthy and had a normal lifespan		To Engineer a mice model suitable to test HER2 vaccines	Animal were immune tolerant to human HER2	Piechocki et al., 2003
MMTV-HER2	Adenocarcinoma with areas of solid, tubular, and papillary growth cellular polymorphism with mitosis and lung metastasis	28 weeks	To study the effectiveness of early and prolonged treatment with the murine form of trastuzumab/Herceptin in MMTV-HER2 model	The study suggested a potential benefit of early treatment with Herceptin in HER2-positive primary breast cancer	Finkle et al., 2004

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-*neu*-NT			Virus-like replicon particles of Venezuelan equine encephalitis virus containing the gene for HER2/*neu* were tested by an active immunotherapeutic approach	Breast cancer growth and tumorigenesis was inhibited	Wang et al., 2005
Pbsn-*neu*	Prostatic intraepithelial neoplasia and invasive prostate cancer	1-2 years	To investigate the implications of HER2 in human prostate cancer	HER2 alone does not play a significant role, but its heterodimerization with HER3 promotes tumorigenesis	Li et al., 2006
MMTV-*neu*-NT on Balb/c			Efficacy of an adenovirus vaccination against *neu* ocongene	The vaccination gave was useful in preventing tumorigenesis	Gallo et al., 2007
MMTV-*neu* (wild type)			To identify tumorsphere forming unit (TFU) and tumour-initiating cells (TIC) in the transgenic model	TFU-based screens can be useful to target tumour-initiating cells in HER2+ breast cancers	Liu et al., 2007
MMTV-*neu* (wild type)			To investigate the antitumor activity of brassins (natural products derived from plants)	Brassin might be useful as the structural basis for a new class of compounds with *in vivo* anticancer activity mediated through the inhibition of 2,3-dioxygenase	Banerjee et al., 2008
MMTV-NIC			To explore the *in vivo* significance of ShcA gene during mammary tumorigenesis	It was demonstrated that signalling downstream from the ShcA adaptor protein is critical for breast cancer development	Ursini-Siegel et al., 2008
MMTV-*neu*-NT on Balb/c background			To investigate the antitumor immunity generated following CD11c-targeted protein vaccines	Delivery of tumour antigens to DCs induced potent immune responses and had antitumoural activity	Wei et al., 2009

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
PTNE-deficient - MMTV-NIC			To address the biological impact of conditional inactivation of PTEN on ErbB-2-induced mammary tumorigenesis	Disruption of PTEN accelerated the formation of multifocal and highly metastatic mammary tumours – such mice may be a valuable tool to test the efficacy of treatments targeting PTNE and HER2	Schade et al., 2009
D16HER2- LUC			To examine the ability of Δ16HER2 to transform mammary epithelium *in vivo* and to monitor Δ16HER2-driven tumorigenesis in live mice		Marchini et al., 2011
MMTV-*neu*-NT	Multiple tumours involving the entire mammary epithelium	18-20 weeks of age in 35-50% of mice	Testing anticancer drug 17-allylamino-17-demethoxy-geldanamycin (17-AAG; tanespimycin), which inhibits the molecular chaperone HSP90		Rodrigues et al., 2012
MMTV-*neu* (wild type)			To investigate a marker to predict outcome of targeted HER2 treatments	17-gene Her2-TIC-enriched signature (HTICS) resulted to be a powerful prognostic that can be used to identify high risk patients that would benefit from anti-HER2 therapy	Liu et al., 2012b
MMTV-*neu* (wild type)			To identify and characterize the putative TICs in the MMTV-Her2/*neu* transgenic mouse model	A technique was developed to highly enrich TICs from mammary tumours of MMTV-*neu* mice, unravelled their properties and identified the cooperative integrin β3- TGFβ signalling axis as a potential therapeutic target for HER2-induced TICs	Lo et al., 2012

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-NIC; ablation of p110a and p110b	MMTV-NIC;p110a revealed 95% survival at 225 days		To elucidate *in vivo* roles of two commonly expressed class Ia phosphatidylinositol 3 kinase (PI3K) in oncogenic receptor signalling	p110b-based was demonstrated to have a regulatory role in receptor-mediated PI3K activity and p110a was identified as an important target for treatment of HER2-positive disease	Utermark et al., 2012
MMTV-*neu* (wild type)			To suggest a guideline for development of prognostic signatures and discuss future directions		Liu et al., 2013
MMTV-*neu* (wild type)			To predict drug responsiveness	Murine-derived gene signatures can predict response even after accounting for common clinical variables and other predictive genomic signatures, suggesting that mice can be used to identify new biomarkers for human cancer patients	Usary et al., 2013
MMTV-HER2; MMTV-rtTA; TetOp-HA-PIKCA^{H1047R}	Rapidly growing, large, multiple tumours; increased angiogenesis, lung metastasis; epithelial-mesenchymal transition; mammospheres	76 days	To investigate the role of aberrant activation of the phosphatidylinositol 3-kinase (PI3K) pathway in diminishing response to HER2-directed therapies	PIK3CA^{H1047R} accelerates HER2-mediated breast epithelial transformation and metastatic progression, alters the intrinsic phenotype of HER2-overexpressing cancers, and generates resistance to approved combinations of anti-HER2 therapies	Hanker et al., 2013
MMTV-*neu*-NT on FVB background			Comparison of prophylactic and therapeutic immunisation with an HER2 fusion protein and immunoglobulin V-gene repertoire analysis	100% effective as a prophylactic treatment, but ineffective as a treatment	Mukhopa-dhyay et al., 2014

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-*neu*-NT on Balb/c background			Review article focusing on the role of tumour microenvironment, oncoantigens and antitumor vaccinations	A better understanding of antigens critical for cancer outbreak and progression, and of the mechanisms that regulate the interplay between cancer and stromal cell populations is needed to develop anticancer strategies	Conti et al., 2014
MMTV-*neu* (wild type) on FVB background			shRNA Kinome screening of targets for HER2 breast cancer therapy	TANK-binding kinase 1 (TBK1) may be a therapeutic target of HER2+ breast cancers in combination therapy	Deng et al., 2014
MMTV-HER2Δ16	Tubular adenocarcinoma consisting of outer pale cells, an intermediate darker fusiform cells, and an inner zcells with pinkish cytoplasm	17 weeks	To better understand the impact of d16HER2 on tumour pathobiology and therapeutic response	d16HER2 revealed to be a signalling axis for decreased risk of relapse after trastuzumab treatment	Castagnoli et al., 2014
MMTV-*neu*-NT			To study HER2 acquired resistance to target drugs	Acquired resistance to HER2 inhibitors might be mediated, by HER4	Canfield et al., 2015
MMTV-*neu*-NT on Balb/c, and transgenic IL-15 knockout (IL15KO/MMTV-*neu*-NT)			To investigate the role played by IL-15 in HER2/*neu* driven mammary carcinogenesis and in its immunoprevention	IL-15-regulated NK and $CD8^+$ memory cells played a role in long-lasting immunoprevention	Croci et al., 2015
MMTV-*neu*-NT			To evaluate the intradermal electroporation to deliver a HER2/*neu* DNA vaccine	Intradermal EP vaccination method revealed to be promising	Lamolinara et al., 2015
MMTV-*neu* (wild type)			To evaluate if mda-7/IL-24 could suppress mammary tumour development	IL-24 might be used for prevention/treatment of human breast cancer	Li et al., 2015

Mouse model	Phenotype/ Pathology	Mean latency of tumours	Use	Main studies outcomes	Reference
MMTV-NIC PTEN on FVB background			The use of this transplantable tumour model provided a powerful preclinical tool with which to test potential novel drug combinations in resistant tumours		Creedon et al., 2016

Table 6.1: *Some published articles from the 1980s to date using transgenic mouse models engaging HER2/neu. The outcomes of researches are briefly summarized.*
**In light blue the model used in the study presented in the following Chapter.*

6.7 Preclinical vs Clinical Oncology

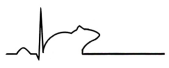
There is urgent need to improve reproducibility and translatability of preclinical data in oncology. Even if during the last two decades several new cancer drugs were approved, built on robust preclinical data, the inability of industry and clinical trials to validate results from the majority of publications on potential therapeutic targets suggests a systemic problem (Begley & Ellis, 2012). Since there is high need in oncology of new drugs, the low success rate of clinical trials can be in part explained by the fact that a large number of drugs with not optimal preclinical data enter them. However, this is not the only reason.

Drug development abundantly relies on literature, therefore good quality published data is essential. Unfortunately, published data was demonstrated to be not always reliable, even when found on prestigious journals (Prinz et al., 2011). Several reasons for the lack of reproducibility have been listed, such as incorrect statistical analysis, insufficient sample size, and competition among laboratories (Prinz et al., 2011). The "published or perish" culture in academia could be conflicting with the integrity of research. Competitive academic environments were demonstrated to increase both scientists' productivity and their bias (Fanelli, 2010). When willing to confirm research findings, only a 11% reproducibility was achieved, despite the efforts to overcome technical differences (Begley & Ellis, 2012).

Apart from these reasons, in particular for oncology, many drawbacks can be found for animal models. For example, xenograft models lack the broad molecular transformation events that occur in human tumours. Also, the animal stromal component of the tumour is a different microenvironment compared to the one in humans, and has an effect on drug response which is often not reflective of the primary tumour. Genetically-engineered mouse models circumvent some limitations as they can, for example, be immune competent, but they still suffer from having rodent-derived stroma (Hutchinson & Kirk, 2011). Nonetheless, even if the models might be immune competent, mice might not have the same elevated levels of circulating immunosuppressive cytokines and various immunological checkpoints, explaining why some promising cancer vaccines did not give the expected results in clinical trials (Mak et al., 2014). Some targeted agents have lower attrition rates compared to non-targeted agents (Hutchinson & Kirk, 2011), even if in some cases also targeted molecular approaches did not result in clinical efficacy despite remarkable success in mice (Mak et al., 2014).

Many aspects can be considered responsible for the high attrition rate from preclinical to clinical studies. Despite everything, animal models have been and are still considered to be

fundamental before new drugs can enter clinical trials. Scientists operating in preclinical studies, can help preventing further drug failures by identifying the correct drug target, using appropriate preclinical models (Hutchinson & Kirk, 2011), evaluating the reproducibility of the results in different models and in different settings. Lastly, it is fundamental to integrate results from different models in order to gather complete data before starting clinical trials.



Chapter 7

Oncolytic Virus-Mediated Immunotherapy in WAP-HER2 Transgenic Mice

Preliminary Testing

7.1 Objectives

Oncolytic viruses (OVs) and OV-mediated immunotherapy are emerging as promising approaches in human patients in the context of targeted cancer treatment.

Introduction of a fully replicant-competent oncolytic virus into a tumour for which the virus is highly specific theoretically allows the spread of the virus, cell deaths and/or lysis, and escape of progeny virions that can go on to infect other surrounding tumour cells in subsequent waves. Incorporating immune stimulatory transgenes into the virus genome may also improve long-term antitumor immunity by directing the immune responses to the tumour.

The aim of this study was to evaluate the use of immune competent C57BL/6 mice, engineered to be tolerant to the human HER2 receptor (therefore able to develop HER2-positive tumours) and that don't develop spontaneous tumours, in order to study the efficacy of retargeted oncolytic viruses that can selectively enter in tumour cells that express such receptor. The clinical impact of the HER2 oncogene is supported by the fact that it is overexpressed in human breast and ovary carcinoma, and in a number of other cancers. HER2 is currently a drug target in oncotherapy.

To validate tolerance of $HER2^{+/-}$ mice, we first demonstrated the ability of two cancer murine cellular lines transduced to express HER2, previously selected through *in vitro* testing, to grow in such transgenic mice. Then, two HER2 retargeted herpes viruses with oncolytic activity, one of them also expressing IL-12, were injected in the subcutaneous tumours of such animal model. Short term and long term systemic immunity was assessed administering challenge injection of cancer cells in animals that survived following OV treatment of the primary tumour.

Such study was possible only thanks to the collaboration with Professor Campadelli-Fiume and her research group, in particular Dr Leoni, from the Department of Experimental, Diagnositic and Speciality Medicine (DIMES), Alma Mater Studiorum – University of Bologna.
This study represents the rationale for future research using this animal model in oncolytic virus-mediated immunotherapy, and other investigations are to date ongoing at DIMEVET in collaboration with DIMES.

7.2 Materials and Methods

All *in vivo* studies were performed at the Laboratory Animal facility of the Department of Veterinary Medical Sciences (DIMEVET), Alma Mater Studiorum – University of Bologna, with the authorization of the experimental protocol by the Italian Ministry in compliance with the Legislative Decree 26/2014 (Protocol number 738/2016 released on July 7th, 2016). SOPs followed are the same of the ones in **Chapter 4.2**.

7.2.1 HER2 Mouse Model

HER2 transgenic mice were bought from the Jackson Laboratories through Charles River (stock number 010562). Heterozygous females and males between 8 and 12 weeks of age were employed for the experiments. The use of HER2 transgenic mice, constructed as described by Piechocki et al. (2003), was necessary since such human receptor would be recognized as *non-self* by the mouse immune system and consequently neoplastic cells growth expressing HER2 would be hindered. The 3 R Principle was followed during the planning of the *in vivo* experiment and during the study itself. Replacement of animals was not possible since no *in vitro* alternative method that can substitute the complexity of an *in vivo* model is to date available in order to evaluate the efficacy of oncolytic viruses, especially considering that the immunological system of the animals are essential for the desired outcomes. Reduction of animals used for the experimental proposes was achieved by the preliminary *in vitro* tests ran at the laboratory of Professor Campadelli-Fiume. The *in vitro* tests allowed to choose the best murine cell lines engineered to express HER2 in which the viruses could effectively replicate and consequentially cause lysis. Furthermore, groups of animals were the smallest possible in terms of animal number still being able to have significance in such preliminary testing when comparing with controls. Finally, special attention was given to the animal's welfare. Refinement was guaranteed through the daily monitoring of the animals and the use of the humane endpoints (**Table 7.1**) to objectively establish pain and distress in mice. If pain was assessed, critical evaluation of the methods available for the alleviation of pain were undertaken.

7.2.2 Oncolytic Herpes Simplex Virus Type 1 (o-HSV)

Two viruses were employed in this study and both of them were engineered at the Laboratory of Professor Campadelli-Fiume by Dr Laura Menotti.

R-LM113 was achieved as described in Menotti et al. (2008). Briefly, such virus was obtained by simultaneously detargeting from nectin1 and HVEM and retargeting to HER2 by moving the site of single-chain antibody insertion at residue 39 and by deleting amino acid residues 6 to 38 (Menotti et al., 2008). The resulting recombinant, R-LM113 (**Fig. 7.1**), was able to selectively enter and spread from cell to cell only via HER2.

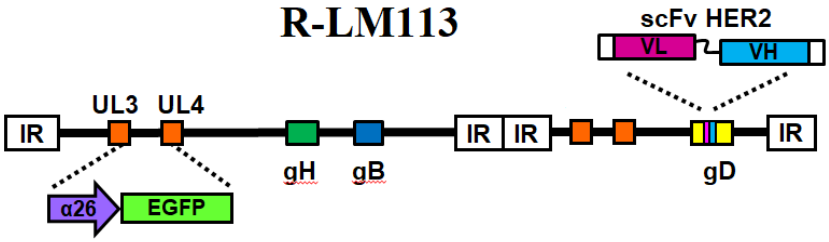


***Fig. 7.1**: R-LM113 Retargeted Herpes Simplex Virus 1 Recombinant Capable of Entering Cells Solely via HER2 (Courtesy of Proessor Campadelli-Fiume).*

The second virus employed in this study, **R-LM113-mIL-12,** is the same of R-LM113 but engineered to express murine interleukin 12 (IL-12), and designed to induce local and systemic antitumor immunity and favour a Th1 response (**Fig. 7.2**).

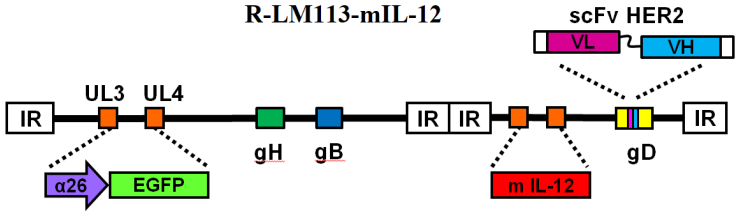


***Fig. 7.2**: R-LM113-mIL-12 Retargeted Herpes Simplex Virus 1 Recombinant Capable of Entering Cells Solely via HER2 and expressing interleukin 12 (Courtesy of Professor Campadelli-Fiume)*

7.2.3 Preliminary *In Vitro* and *In Vivo* Tests

At the Laboratory of Professor Campadelli-Fiume, four mice tumour cell lines were transfected with lentivurs in order to express HER2 (line A-HER2, Line B-HER2; Line C-HER2; Line D-HER2). Viral replication on all tumour cell lines for both R-LM113 and R-LM113-mIL-12 viruses was evaluated at 24 and 48 h after inoculation and measured as plaque forming unites per millilitre (PFU/mL). Virus replication was satisfactory for lines A-HER2 and B-HER2 which were

therefore selected for *in vivo* studies (**Fig. 7.3**), even though apparently both viruses showed a 1 to 2 log reduction in PFU/mL in line B-HER2 compared to line A-HER2.

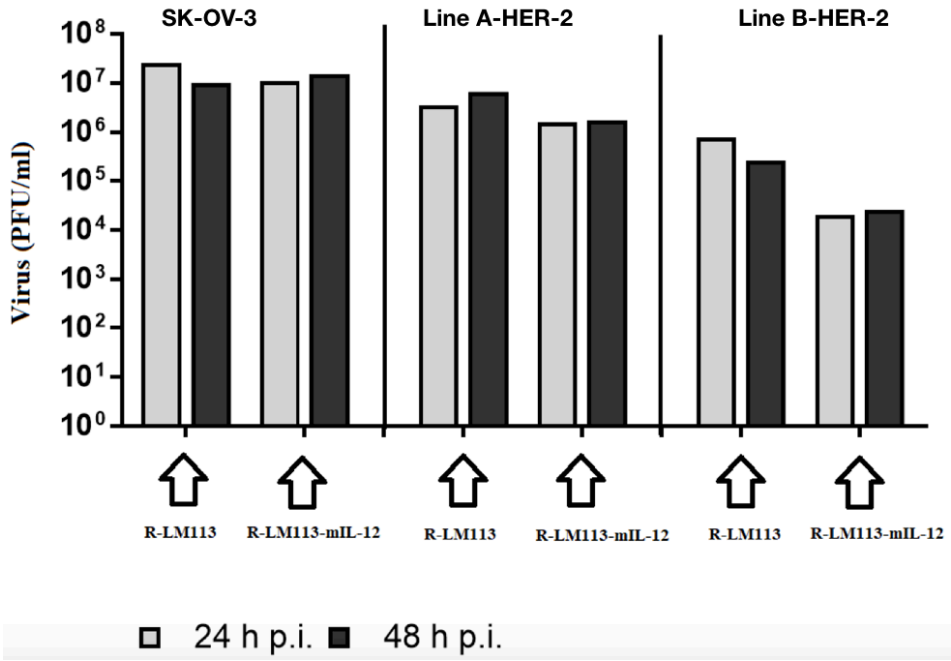


***Fig. 7.3**: R-LM113 and RLM113-mIL-12 replication in mice tumour cell line A-HER2 and line B-HER2 compared to control (SK-OV3). Values expressed as PFU/mL at 24 and 48 h after inoculation (Courtesy of Professor Campadelli-Fiume).*

Consequentially, a preliminary *in vivo* test was conducted. The cell lines (A-HER2 and B-HER2) were administered subcutaneously (300 µL of PBS containing $2x10^5$ cells) in HER2 transgenic mice. When the tumour was appreciable, R-LM113 was injected intratumorally and animals were sacrificed at 24 and 48 h after injection. The observation of tumours in fluorescence revealed that *in vivo* replication of the virus in cell line B-HER2 was lower than in cell line A-HER2, differently from what was evidenced by *in vitro* testing. Therefore, only cell line A-HER2 was chosen for further *in vivo* testing.

7.2.4 Housing and Breeding

Housing conditions (temperature, humidity, light, cages, litter, environmental enrichment) were the same of **Paragraph 4.2.2**.

Mouse breeding was started from 12 HER2 heterozygous and 9 wild type mice. Animals were kept in quarantine for 10 days prior entering the facility. Breeding pairs were made of 2 wild type females and 1 heterozygous male, mated just after weaning and until dams were able to deliver successfully. Pups were weaned between 25 and 30 days of age, caged in sex groups

and identified using the headset marking as described in **Paragraph 4.2.2** (**Fig. 4.2**). Tissue samples deriving from the tail tip or from the ear during the headset marking were used for genotyping.

7.2.5 Genotyping

Genotyping was conducted at Professor's Campadelli-Fiume laboratory, at the Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna. DNA was extracted from mouse tail or ear sample following standard procedures using Nucleo Spin® Tissue (Machery-Nagel) kit and analysed in PCR with the following set of primers: HER2 forward 9735GAGCCGCGAGCACCCA, and reverse 9736GGTGGGCAGGTAGGTGAGTTCC; internal positive control forward oIMR7338CTAGGCCACAGAATTGAAAGATCT, and reverse oIMR7339GTAGGTGGAAATTCTAGCATCATCC. DNA fragments were separated by electrophoresis on agarose gel.

7.2.6 *In Vivo* Testing

Before any experiment was started, animals were shaved on the left flank or on the right flank to better identify the injection area for establishment of primary tumour or challenge, respectively. Every injection (tumour cells or viruses) was done with the mice under anaesthesia with 2.5% isoflurane in O_2 (as described in **Paragraph 4.2.7**).

Study 1. R-LM113 vs R-LM113-mIL-12

Thirty-three anesthetized mice were injected subcutaneously (SC) with 300 µL of PBS containing $2x10^5$ cells of line A-HER2 on the left flank (**Fig. 7.4**) at day 0.

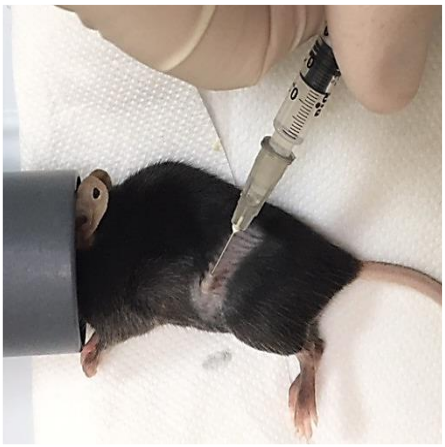


Fig. 6.4*: Anesthetized mice receiving subcutaneous injections of tumour cells transfected with lentivirus in order to express HER2 (line A-HER2).*

The experimental groups were the following ones.

Group 1: Control group. Thirteen animals (6 females and 7 males) were used as controls and received 300 µL PBS.

Group 2: R-LM113 group. Ten animals (3 females and 7 males) were injected with 300 µL of PBS with $1x10^8$ PFU/mice of R-LM113.

Group 3: R-LM113-mIL-12 group. Ten animals (6 females and 2 males) were injected with 300 µL of PBS with $1x10^8$ PFU/mice of R-LM113-mIL-12.

Animals were treated at days 3, 6, 12, 18 post implantation (p.i.). Therefore, the first treatment (3 days p.i.) started before the mass was appreciable. When the mass was present, treatment was administered intratumorally, otherwise it was administered subcutaneously in the injection area. Mass size was registered at days 6, 12, 18, 21, 26, 35, 40 p.i.

Study 2. Early vs late treatment with R-LM113-mIL-12

Part A)

Twenty-two anesthetized mice (groups 1, 2, and 3) were injected SC with 300 µL of PBS containing $2x10^5$ cells of line A-HER2 on the left flank (day 0).

The experimental groups were the following ones.

Group 1: Control group. Six animals (4 females and 2 males) were used as controls and received 300 µL PBS.

Group 2: Early treatment group. Eight animals (5 females and 3 males) were injected with 300 µL of PBS with $1x10^8$ PFU/mice of R-LM113-mIL-12 starting from day 3 p.i..

Group 3: Late treatment group. Eight animals (4 females and 4 males) were injected with 300 µL of PBS with $1x10^8$ PFU/mice of R-LM113-mIL-12 starting from day 10 p.i..

Group 1 and Group 2 were treated at 3, 7, 10 and 14 days p.i..

Group 3 was treated at 10, 14, 17 and 21 days p.i..

When the mass was present treatment was administered intratumorally, otherwise it was administered subcutaneously in the injection area.

At day 18 p.i. animals that hadn't reached the humane endopoint undergone a second injection (challenge) of $2x10^5$ cells of line A-HER2 on the right flank. The use of the challenge had the aim of simulating the growth of a metastatic tumour and was used to evaluate if the treatment of the primary tumour with the virus was able to develop a protective immune response. For such challenge, the following control group was used.

Group 4: Challenge control group. Six mice (4 females and 2 males) received $2x10^5$ cells of line A-HER2 on the right flank at day 18.

Part B)

Some animals from Study 2, who hadn't developed the mass in part A of the study, received a second challenge injection of $2x10^5$ cells of line A-HER2 on day 82, always on the right flank. In particular, groups were the following ones:

Group 1. Second challenge control group. Such group was made of five animals (3 females and 2 males) receiving tumour cells for the first time. These animals age-matched group 2.

Group 2. Second challenge group. Such group was made of seven mice, five mice of which (3 females and 2 males) from the early treatment group of part A, and two mice (1 female and 1 male) from late treatment group of part A.

Such challenge was needed to evaluate if the protective action was able to be long-term effective.

7.2.7 Humane Endpoints

Study endpoints are essential in biomedical research using animal models, and must be addressed for every animal in the study. We used a standardized score table to evaluate adverse effects, pain or distress for each animal (**Table 7.1**). The effective use of such endpoints required properly trained and qualified operators performing both general and study specific observation of the research animals at appropriate time points.

Parameter	Animal Condition	Score
Feature	Normal	0
	Poor grooming, index of mild depression of the sensorium	1
	Matted fur	2
	Significant loss of fur, curved posture	3
	Lateral or abdominal decubitus or limb/limbs paralysis	4
Intake of food and water	Normal – Unknown: body weight < 5%	0-1
	Total anorexia: body weight < 15%	2
	Cachexia: poor general condition and evident weight loss	3
Respiratory symptoms	Normal respiratory rate	0
	Slight alterations of the respiratory rate	1
	Increased respiratory rate and abdominal breathing	2
	Decreased respiratory rate speed and abdominal breathing	3
	Marked abdominal breathing and cyanosis	4
Spontaneous behavior	Normal	0
	Slight alterations; excitability	1
	Decreased mobility and alert; solitary confinement	2
	Restless or very still; compulsive behaviours; circling, (repeated circular movements) as index of brain suffering	3
Induced behavior	Normal	0
	Mild sensorium depression or exaggerated response to stimuli	1
	Moderate changes in typical behavior	2
	Violent or extremely low reaction	3
Additional parameters	Rotated ears outwards and/or back; sharpened snout; narrow and half-closed eyes	4
	Mass sizes bigger than 1500 mm^3, ulcerated or that interfere with the animal ability of eating and drinking*	4
	Abdominal distention associated with fluid pressure causing 10% increase in weight*	4
	Subcutaneous tumour ulceration exceeding the size of 2 mm	4
Total Score		

Table 7.1*: Standardized score table to evaluate animal suffering and define the humane endpoints. Experimental humane endpoints: the animal must be sacrificed when it reaches the score 10 of the table. The animal must be immediately sacrificed if it presents one of the signs evaluated with the highest score for a specific sign 4. The total score is classified as: 0-4 = Normal; 5-9 = Needs daily monitoring; 10 = Animal with initial distress signs; 11-13 = Animal with distress signs; ≥ 14 = severe distress.*

The increase in weigh was not considered to be a reliable parameter to detect animal's suffering since muscular atrophy or malnutrition caused by the subcutaneous tumour could have theoretically masked the increase of weight caused by the mass size. The mass size was considered to be a more reliable factor, together with the body condition score (BCS) (**Fig. 7.5**).

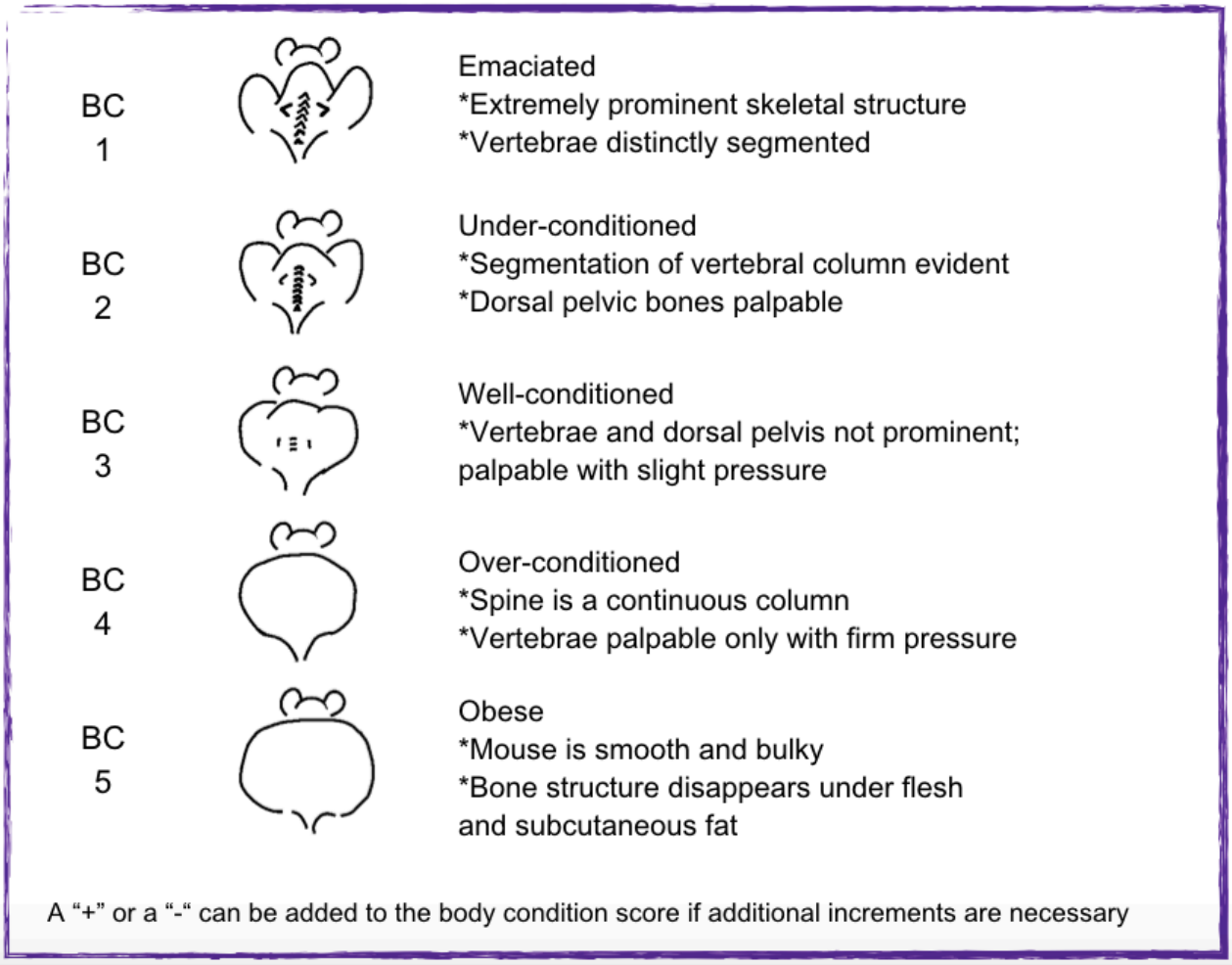


***Fig. 7.5**: Body condition score (adapted from Ullman-Culleré & Foltz, 1999).*

The most important parameter taken into consideration was the mass volume. In fact, being the tumour subcutaneous, no general symptoms were expected. However, animals were constantly closely observed.

The mass was assessed twice weekly by its measure using a digital caliper (Digital Reading Stainless Steel Vernier Caliper, 150±0.02mm, Biological Instruments, Besozzo, Varese, Italy) while mice were conscious. The two longest perpendicular axes in the x/y plane of each tumour were measured by always the same operator familiar with collecting caliper

measurements of tumours in mice. The depth was assumed to be equivalent to the shortest of the perpendicular axes, defined as y. As in standard practice (Euhus et al., 1986; Tomayko & Reynolds, 1989), measurements were calculated according to the following equation:

Tumour volume: $xy^2/2$

Mice were sacrificed when tumour size reached the ethical limit of 1500 mm^3.
If the mass's skin appeared to be thin and red (**Fig. 7.6**) the animal was carefully monitored in order to rapidly detect skin ulceration. If ulceration exceeded 2 mm, was itchy, became necrotic or infected animals were considered to have reached the humane endpoint.

***Fig. 7.6**: **A)** Red and thinned skin at the mass level; **B)** tumour ulceration.*

Finally, if tumours were considered to interfere with eating or impaired ambulation, animals were considered to have reached the humane endpoint.
It has to be noted that epidermis, adipose tissue, fur, as well as human variability in measuring and volume estimation might all contribute in adding imprecision to the mass's volume measurement (Ayers et al., 2010). Nowadays, other techniques, such as ultrasound, are considered to be more precise in this type of experimentation. However, most of the published research articles in this field use digital calipers and measurements were considered to be reliable and adequate for the study.

7.2.8 Statistics

Data were analysed using GraphPad Prism 7 for Macintosh and evaluated through growth curves. Kaplan-Meier with log-rank test was used to study survivals. Statistical significance was determined at the level of $p < 0.05$.

7.3 Results and Discussions

7.3.1 Housing and Breeding

Heterozygous HER2 mice ($HER2^{+/-}$) displayed a normal phenotype and were indistinguishable to their wild type littermates. Genome recognition was possible only through genotyping as described in **Paragraph 7.2.5**. No apparent pathology was observed in all animals, and no mice had spontaneous tumour growth as previously described by Piechocki et al. (2003). Transgenic and wild type C57BL/6 were quite touchy towards operators. Often mice, especially males, caged together needed to be separated because they were aggressive and caused each other fight wounds.

Heterozygous males were good breeders such as wild type female. Mating between heterozygous mice was never performed, since a previous study showed that $HER2^{+/+}$ are embryonic lethal (Yong et al., 2015). In an 8-month period, dams showed a mean time between delivery of 34 days. Litters were made of 46,83% heterozygous and 53,17% wild type mice. Females were kept in reproduction for 5-8 months, depending on the needs of the planned experimentation. Mean pup litter size ranged between 1 an 9, with a mean of 5 pups per litter.

7.3.2 *In Vivo* Testing

Among all humane endpoints considered by the protocol, mass volume and ulceration were the only ones positive since animal's conditions always resulted negative for every other parameter considered in **Table 7.1** for humane endpoints. In some cases, cutaneous ulceration occurred, but was always of size < 2 mm and spontaneously healed.

The scientific aims and objectives were accomplished without adverse effects, pain or distress to the animal.

Study 1. R-LM113 vs R-LM113-mIL-12

As it can be seen from **Fig. 7.7**, all animals from the control group (group 1) developed the tumour. Most of them had appreciable masses starting from day 7 p.i.. This provides evidence of the validity of $HER2^{+/-}$ transgenic mice as a model for the development and growth of HER2 cancers and, therefore, the possibility to study innovative targeted treatments for HER2-positive cancers in immune competent animal models.

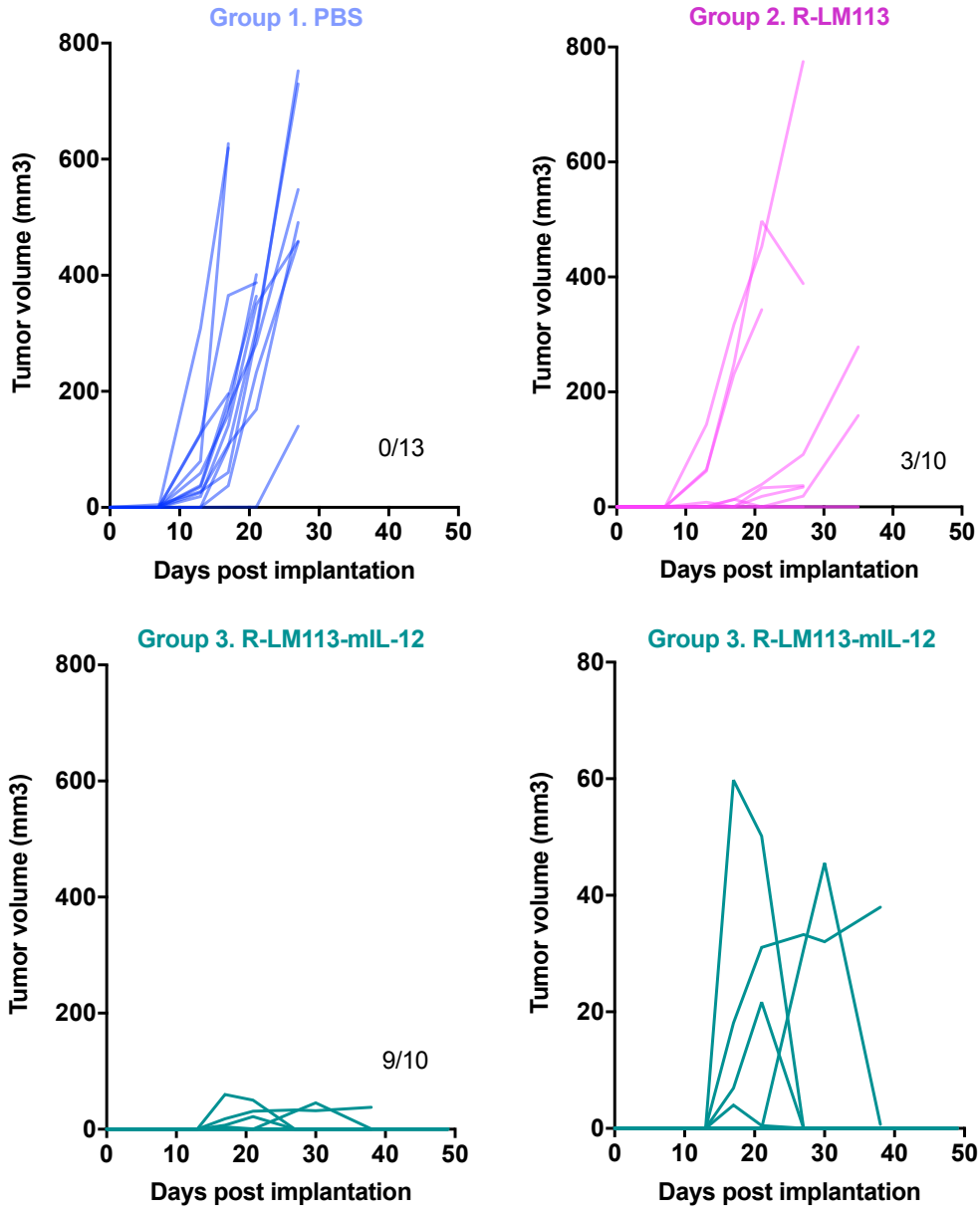


Fig. 7.7: *Spaghetti graph for tumour's growth in group 1 (PBS control), group 2 (R-LM113) and group 3 (R-LM113-mIL-12). No animals were tumour free at the end of the observation time for group 1, three animals out of ten were tumour free for group 2, and, nine animals out of ten were tumour free in group 3.*

In group 2 receiving R-LM113, the tumour's growth was inhibited in three animals which were negative for the presence of the mass at the end of the observation time. Four animals showed

reduced tumour growth compared to controls and such growth was delayed compared to group 1. However, three animals out of ten showed a trend similar to that seen in group 1.
On the other hand, nine mice out of ten of group 3, receiving R-LM113-mIL-12, were negative for the tumour at the end of the observation time. Five animals had small masses (between 20 and 80 mm^3 in size) which arose delayed (day 12-22 p.i.) compared to group 1. In four of these five animals, the mass rapidly disappeared and the remaining one had a small mass (40 mm^3) on day 40 p.i.

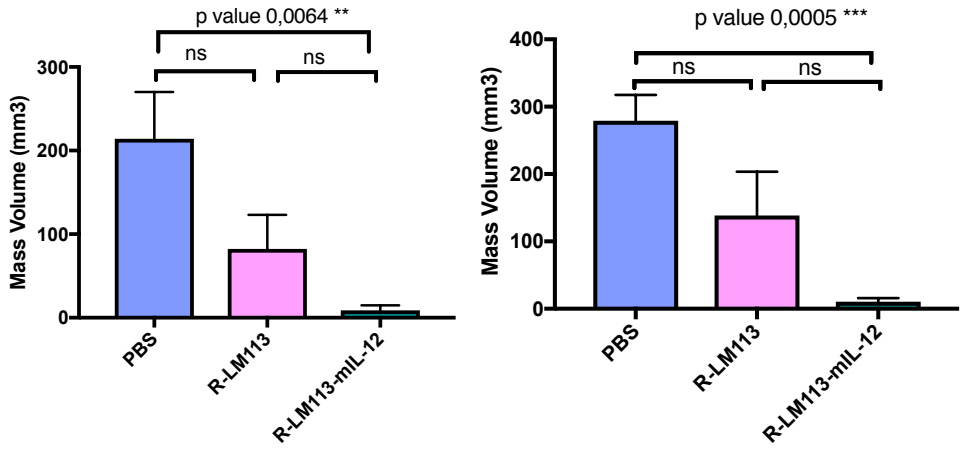


***Fig. 7.8**: Tumour mass volume on day 17 (left) and on day 21 (right) p.i.*

As it can be seen from **Fig. 7.8**, the different trend in tumour's masses volumes seen in group 2 was not significant on days 17 and 21 p.i., due to the effect of the three animals who developed the tumour in a similar way to the controls. On the other hand, differences in volumes were significant ($p < 0.05$) between group 1 and group 3 (**Fig. 7.8**).
The first virus administration occurred before any mass could be detected. R-LM113-mIL-12 revealed to be better than R-LM113 in preventing HER2-positive mass growth and showed an increased oncolytic activity presumably attributed to the expression of IL-12. This study underlines how *in vivo* studies are essential. In particular, *in vitro* tests showed a better replication of R-LM113 compared to R-LM113-mIL-12 but this was the opposite in the *in vivo* study. In accordance with previous studies, this provides evidence of the main role played by the immune system as an effector of antitumor therapy and underlines the need to conduct such preclinical research in immune competent animals, since *in vitro* strategies are not able to reproduce the complexity of the *in vivo* immune system.
Because of the results of study 1, we pursuit studying only R-LM113-mIL-12 in the following study.

Study 2. Early vs late treatment with R-LM113-mIL-12

Part A)

In study 1 we assessed that line A-HER2 cells start forming a mass around day 7 p.i. Therefore, we studied the difference in oncolytic activity of R-LM113-mIL-12 when administrated early (when the mass is yet not present, i.e. 3 days p.i.) or late (when mass is usually already palpable, i.e. 10 days p.i.).

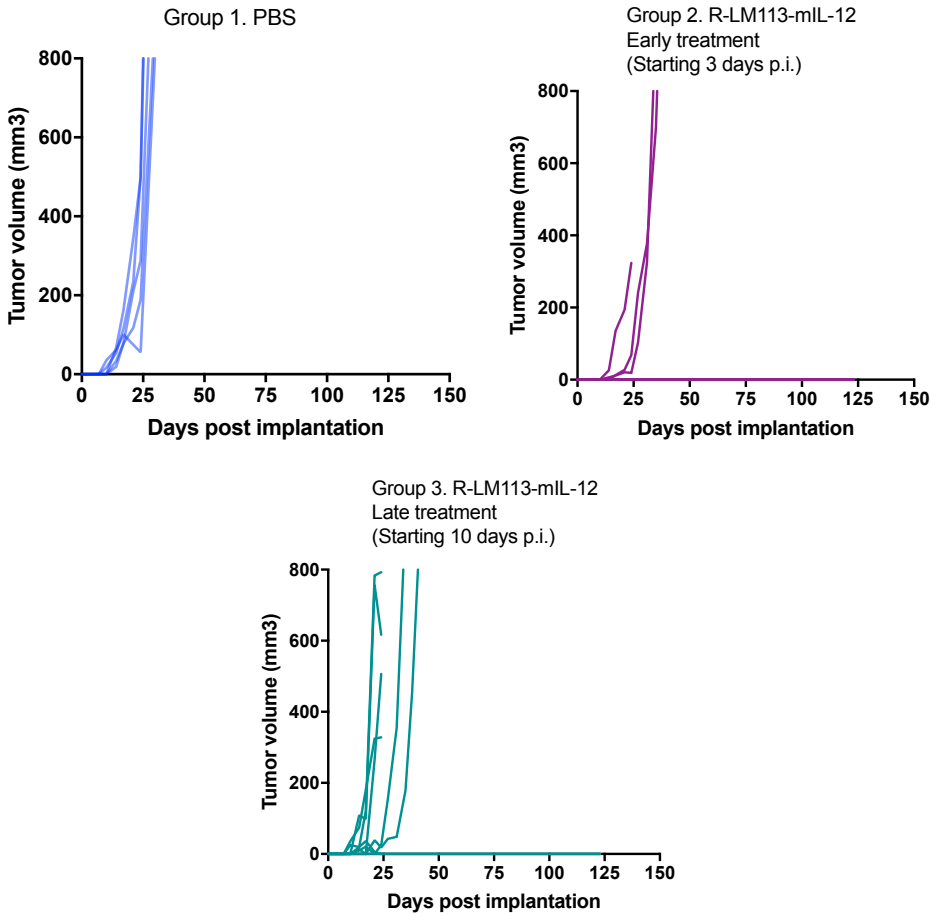


Fig. 7.9: *Spaghetti graphs for tumour's growth in group 1 (PBS control), group 2 (R-LM113-mIL-12 starting from 3 days p.i.) and group 3 (R-LM113-mIL-12 starting 10 days p.i.). No animals were tumour free at the end of the observation time for group 1, five animals out of 8 were tumour free for group 2, and two out of 8 were tumour free in group 3.*

As seen in **Fig. 7.9** all animals from group 1 showed a fast and exponential growth of the tumour in accordance with **Study 1**.

Five out of eight animals from group 2, receiving early treatment with R-LM113-mIL-12 haven't developed a mass during all the study. In the three animals with the mass, one showed a fast tumour growth and was sacrificed at 25 days, while the other two reached the humane

endpoint for mass size and were sacrificed at days 38 and 45 p.i.. However, these two mice underwent the challenge injection on day 18 p.i. (see below).

Late treatment in group 3 inhibited tumour's growth only in 2 animals out of eight. Of the six animals with cancer growth, four were sacrificed at day 24 p.i because mass sizes reached the humane endpoint, while the remaining two although having a delayed growth were sacrificed at day 38 and 45 p.i., respectively, however receiving the challenge injection on day 18 p.i. (see below).

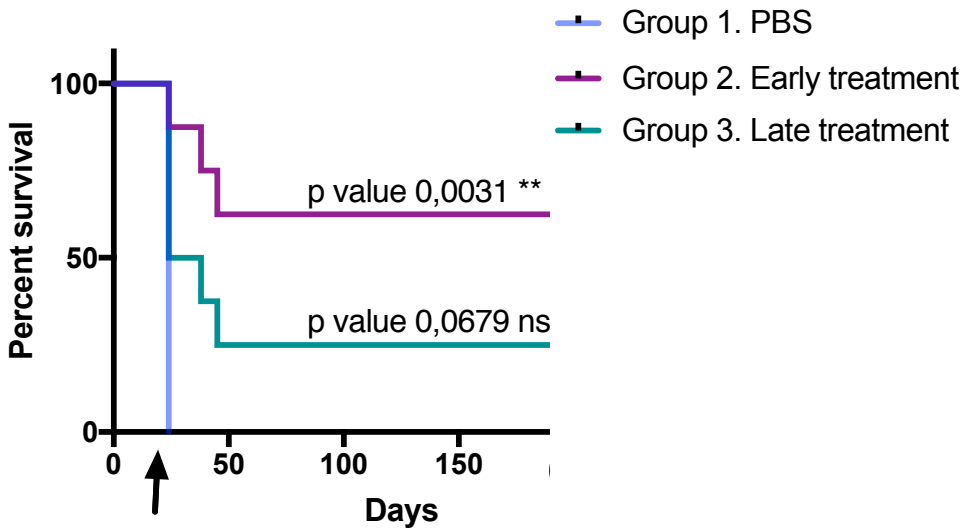


Fig. 7.10: *Kaplan Meier survival plot for group 1 (n=6), group 2 (n=8) receiving early treatment, group 3 (n=8) receiving late treatment. The arrow indicates challenge injection of line A-HER2 cells on day 18 post primary tumour implantation.*

Apparently, both treatments provided improved survival rates compared to the controls (**Fig. 7.10**). However, percent survival was significant ($p < 0.05$) compared to the control group only when the early treatment with R-LM113-mIL-12 was provided (**Fig. 7.10**).

On day 18 p.i. all animals from group 2 and group 3 who were alive and hadn't reached the human endpoint received a challenge injection of line A-HER2 cells on the right flank.

The new control group (group 4), who never received tumour cells injections neither virus injection before, showed a normal growth of the mass as previously described in control groups.

Of the seven animals from group 2 receiving the challenge injection none of them showed tumour's growth on the right flank. Two animals from this group were however sacrificed at day 38 and 45 p.i., respectively, because the size of the primary tumour reached the humane endpoint. It is to note that during the 10 and 17 days, respectively, after the injection of the

challenge in such two animals, no growth was registered for the challenge tumour. Therefore, it is fair to think that an adaptive immunity against line A-HER2 cells was developed in these two animals.

Two of the four animals of group 3 surviving the primary tumour at day 18 p.i. and receiving the challenge never developed any mass. The remaining two mice were sacrificed at day 38 and 45 p.i. because the size of the primary tumour reached the humane endpoint, but no secondary mass developed.

Obviously, these differences between group 1 and 2 and between group 1 and 3 were significant ($p < 0.05$) (**Fig. 7.11**).

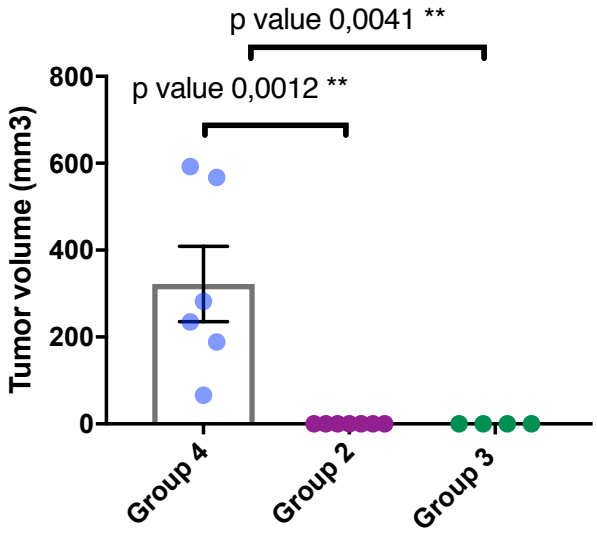


***Fig. 7.11**: Comparison between groups in challenge's growth, measured on day 38 p.i.*

Part B)

The animals who survived Study 2 - part A undergone a second challenge injection on day 82.

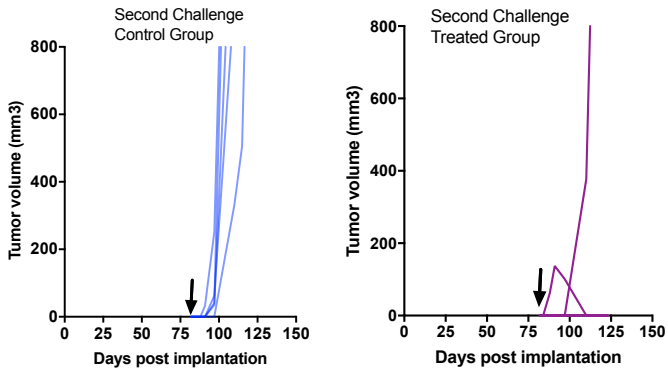


***Fig. 7.12:** Tumour's growth following second challenge injection.*

All animals from the control group developed a mass. Of the seven animals tested in part A, only two developed a mass, one of which regressed spontaneously (**Fig. 7.12** and **7.13**). The other animal was sacrificed because the mass reached the humane endpoint.

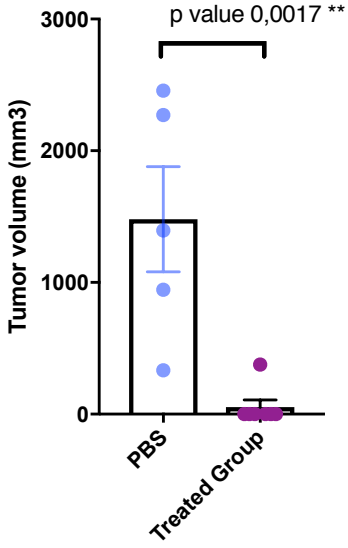


***Fig. 7.13**: Comparison between groups in challenge's growth, measured on day 110 p.i.*

These results, underline how the adaptive immunity was long-lasting. It is fair to think, in accordance with previous studies, that $CD8^+$ T cells responding to released tumour-associated antigens are of primary importance for such protective role. The development of this study will have to take into consideration, for example, the presence of tumour infiltrating lymphocytes (TILs), and particularly $CD8^+$ TILs, which were already found to be associated with improved overall survival in some kind of cancers (Sato et al., 2005; Hamanishi et al., 2007; Hwang et al., 2012; Kotoula et al., 2016).

7.4 Conclusions

HER2 mouse model was already demonstrated to be tolerant to HER2-positive tumours, differently from wild type mice who show high levels of immunogenicity against the HER2 antigen (Yong et al., 2015). *Neu*, the rat homologue of the human HER2, has about 10% difference with the human receptor, therefore mice models tolerant for *neu* aren't the best models to study targeted therapies and immunotherapies. In our study, we demonstrated that the transgenic mice well tolerated the growth of tumours deriving from a specific murine cancer line of transduced HER2 cells. Thus, the mouse demonstrated to be a reliable model for such cell line. We also showed, how *in vivo* experimentation is to date essential since *in vitro* models are not always able to simulate the complexity of organisms and not always can predict the experimental outcomes. The HSV-1 viruses tested in this study have the characteristic that they are not attenuated. The specificity for tumour cells was gained by detargeting HSV-1 from its natural receptors and targeting them to HER2, an important receptor overexpressed in a number of cancers and correlated with poor prognosis. R-LM113 is an "unarmed" oncolytic virus which *in vitro* replicated better than R-LM113-mIL-12, which is "armed" with IL-12. However, *in vivo*, R-LM113-mIL-12 gave better results, underlining the importance of the immune system in fighting cancer. This result was not surprising and is in accordance with previous studies that report the need of *in vivo* experiments in immune competent models to better define the role of the immune system in enhancing antitumor activity (Thomas et al., 2016).

Intratumoral injection ensured high-dose delivery to the target, minimized systemic reactions, and limited complement and antibody-activation of the OV (Lawler & Chiocca, 2015). In this preclinical study, the intratumoral injection permitted bypassing the tumour architectural barrier. It has to be noted, that this type of administration is limited to tumours that are physically accessible (subcutaneous in our case) by palpation or direct imaging. However, the immune systemic antitumor response overcame such limitation, as seen with the challenge experiments. In fact, the viruses were injected directly intratumorally, entered the tumour cells, replicated causing cell death and/or lysis, and progeny virions escaped to the surrounding cells in subsequent waves (Lawler & Chiocca, 2015). Nonetheless, a potent antitumor T-cell response was likely to have occurred, and not only the infected tumour was eliminated but also challenge tumours growth was inhibited after 18 and 82 days, showing an important adaptive immunity response.

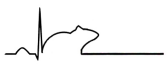
The most important conclusion that can be drawn from our results is that repeated administration, starting when the mass is still not palpable, of both R-LM113 and R-LM113-mIL-12 viruses resulted in a high percentage of tumour free mice for the time interval of examination. The mice receiving the viruses but that we not tumour free, showed however an evident delayed tumour growth. When expressing mIL-12 the virus was more effective. Furthermore, animals who did not develop the primary tumour after early and late treatment with the oncolytic virus expressing mIL-12, were protected from the onset of a challenge tumour, after 18 and 82 days.

IL-12 is one of the most important anticancer cytokines and has been demonstrated to enhance anticancer immune response. Its systemic administration to cancer patients causes excessive clinical toxicity and severe side effects (Lasek et al., 2014). Different methods are being tested by scientist in order to decrease IL-12 systemic toxicity, or in order to deliver it directly intratumorally (Tugues et al., 2015). Taking advantage of OV oncotropism and the wide genome platform offered by HSV-1 for manipulation, a virus was engineered in order to deliver IL-12 directly within the tumour environment. No side effects were noted in the preclinical animal model. Safety profiles for oncolytic viruses are usually very high, especially if they have as only target the receptors overexpressed on tumour cells. Once the virus encounters a non-tumour tissue it self-exhausts and cannot revert to wild type tropism. The very tolerable safety profiles of OVs, and their ability to modulate the tumour microenvironment provides a rational strategy for combination treatment with other cancer agents to improve therapeutic responses (Kaufman et al., 2015).

Limitations to the application of such viruses in clinical situations could be represented by the extent of immunosuppression in patients with treated but progressing advanced tumours, which may contribute in decreasing efficacy (Lawler & Chiocca, 2015). Tumour size and heterogeneity can also be a limit to virus biodistribution, as also hypoxic environments (Kaufman et al., 2015). For example, in our study, when treatment was initiated late and when tumour mass was already detectable, percent survivals were lower. Furthermore, the overall effectiveness of OVs can depend on the susceptibility of cancer cells to undergo apoptosis. In fact, if this happens too rapidly, the virus doesn't have the time to replicate and spread within the tumour and the amount of active virus within it decreases and might not be sufficient.

Finally, following such preclinical testing, these innovative therapies should be considered for clinical trials even in dogs and cats with HER2-positive cancers. Constructing an oncovirus on the canine herpesvirus (CHV-1) might improve replication and infectivity of the virus.

In conclusion, HER2 immunocompetent transgenic mice are an ideal system to investigate the activity of OVs that are engineered to target specifically human HER2, both if they are "unarmed" or "armed" with cytokines. Such model is able to develop HER2 cancers and gives the possibility of investigating new targeted therapies in which the immune system has an important role. Our translational pilot study provides preliminary evidence supporting that non-attenuated retargeted oHSV-1 might be an effective treatment agent for HER2-positive cancers.



Chapter 8

Final Conclusions

Biomedical research has changed substantially in the last few decades thanks to complementary methods that are able to partially substitute the use of laboratory animals. In order to test new treatments in human/animal clinical trials the use of laboratory animals is unfortunately still a law requirement since no alternative method can mimic such complex biological systems. Furthermore, it would be unethical to test directly on humans or domestic animals. Fortunately, standards for animal care have increased, and authorities, scientists, veterinarians and other members of the laboratory team are taking their obligation seriously to provide the best possible care for laboratory animals, in order to guarantee their welfare. Moreover, it is increasingly evident that the better care provided to laboratory animals produces more reliable research data.

Among all animal models, mice are used most often because of many characteristics that make them close to an ideal model. In particular, genetically engineered mice models have proven to be powerful tools in understanding the mechanisms of genetic diseases. They have also been useful in testing novel treatments. We have seen how C57BL/6 has proven optimal for this purpose and it is one of the strains most employed in biomedical research.

All this is the framework in which the two research projects discussed in this thesis are developed. Both projects were conducted in parallel during the three years PhD course in the Laboratory animal facility of the Department of Veterinary Medical Sciences, University of Bologna. Both studies employed engineered mice models constructed on a C57BL/6 genetic background, and the know-how gained with one project positively influenced the other.

The first research is on a knock-in transgenic model homologous for the genetic cause of Hutchinson-Gilford Progeria Syndrome, a very rare human genetic disease (affecting 1 out of 8 million births) that causes accelerated aging. The model was created by professor Otín in 2011, and a first description was provided in Osorio et al. (2011). When a new genetic line is created, a detailed description of its phenotype must be undertaken. This includes information on how to maintain the line and its phenotype, with details relating to the onset of changes and disease progression, and information on how to manage their welfare effectively. The detailed and structured description of the mouse phenotype also enabled us

to answer in depth the question **"How much does the model resemble the human disease?"**. Similarities and differences between patients and mice were highlighted, and background confounding effects were pointed out. The description will be of use to research groups in programming their studies, both for the breeding and housing of *Lmna*^{G609G} transgenic mice and the use of the model in drug testing. Data collected will be useful also because of the translational potential of the mouse model in understanding the physiological aging process.

Since a combination of low dose of rapamycin (RAPA) and all-trans retinoic acid (ATRA) was successful *in vitro* in reducing progerin, the question **"Do ATRA and low doses of RAPA treatment improve the phenotype of *Lmna*^{G609G} transgenic mice and prolong their lifespan?"** was answered. The dose regimen used in such study was 1 mg/kg of rapamycin combined with 0.4 mg/kg of all-trans retinoic acid, twice weekly, starting from 6 weeks of age. Differences in survival analysis, weight trends and other parameters taken in consideration were not significantly different between treated and untreated groups. However, this was a preliminary study which considered only males, and animal groups were small in number. The doses used in this study were considered to be safe, since animals did not show side effects associated with the drugs. Slightly higher doses, especially of rapamycin, are now being tested in our facility. The collaboration with the National Research Council, Institute of Molecular Genetics, Bologna, will continue and, in addition to the question as to the right dose to use, we will address questions such as whether sex influences the treatment outcomes and the best timing to start the treatment. Moreover, we are studying other promising treatments using the described mouse model.

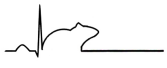
The second research described in this thesis used immune competent C57BL/6 mice, engineered to be tolerant to the human HER2 receptor (overexpressed in a number of human and pet cancers) to study the efficacy of retargeted oncolytic viruses that can selectively enter in tumour cells that express such receptor. The viruses under study were created at the Laboratory of Prof. Campadelli-Fiume (DIMES, Bologna) by Dr Menotti (Menotti et al., 2008), and specifically were R-LM113 and the same virus modified in order to express IL-12 (R-LM113-mIL-12). Following *in vitro* preliminary research conducted at the Department of Experimental, Diagnostic and Specialty Medicine, Alma Mater Studiorum – University of Bologna, two out of four murine cancer cell lines transfected with lentivirus in order to express HER2 were selected for the *in vivo* study. The viruses were able to successfully replicate *in vitro* only in these two cell lines. Further preliminary *in vivo* testing brought about the decision

to test only one murine cancer cell line in which virus replication gave better results as opposed to from what was evident during *in vitro* testing. The research addressed these questions: 1) **"Is the mouse tolerant to the growth of specific cancer murine cell lines transduced to express HER2?"**; 2) **"Which of the two tested oncolytic viruses replicates better in the tumour *in vivo*?"**; 3) **"Is the model able to produce a protective immune response against secondary tumours?"**. The mouse demonstrated to be a reliable model since it enabled the development of HER2 cancer cell lines and allowed the virus to replicate within the tumour. Animals did not show any side effects and the safety profile of the virus was confirmed. R-LM113-mIL-12 gave better results compared to R-LM113, underlining the importance of the immune system in fighting cancer and the importance of an immune competent model in oncological research. R-LM113-mIL-12 was able to produce an adaptive immune response that protected mice from the development of secondary tumours up to 82 days post primary tumour implantation. These results represent the rationale for future research in this field using $HER2^{+/-}$ C57BL/6 as models to investigate the safety and efficacy of oncolytic virus-mediated immunotherapy and show how *in vitro* research is still not sufficient in preclinical testing.

In conclusion, we can state that both animal models considered in this thesis were revealed to be appropriate for testing specific therapies.

It is important to underline that biomedical research using preclinical animal models needs to consider the welfare of the animals essential, both for ethical reasons and for the quality of the research data. Veterinarians are obviously vital for animal care; because of their special training, their contribution is essential during *in vivo* research.

Finally, it should always be kept in mind that any given response in a mouse may not occur in precisely the same way in humans, and that data acquired through *in vivo* preclinical studies should be complemented by *in vitro* and *ex vivo* research. Moreover, it is important to report also negative preclinical outcomes in order to minimize the failure rate of clinical testing based on translation of preclinical data.



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