Cutaneous and metabolic defects associated with nuclear abnormalities in a transgenic mouse model expressing R527H lamin A mutation causing mandibuloacral dysplasia type A (MADA) syndrome

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LMNA gene encodes for lamin A/C, attractive proteins linked to nuclear structure and functions. When mutated, it causes different rare diseases called laminopathies. In particular, an Arginine change in Histidine in position 527 (p.Arg527His) falling in the C-terminal domain of lamin A precursor form (prelamin A) causes mandibuloacral dysplasia Type A (MADA), a segmental progeroid syndrome characterized by skin, bone and metabolic anomalies. The well-characterized cellular models made difficult to assess the tissue-specific functions of 527His prelamin A. Here, we describe the generation and characterization of a MADA transgenic mouse overexpressing 527His LMNA gene, encoding mutated prelamin A. Bodyweight is slightly affected, while no difference in lifespan was observed in transgenic animals. Mild metabolic anomalies and thinning and loss of hairs from the back were the other observed phenotypic MADA manifestations. Histological analysis of tissues relevant for MADA syndrome revealed slight increase in adipose tissue inflammatory cells and a reduction of hypodermis due to a loss of subcutaneous adipose tissue. At cellular levels, transgenic cutaneous fibroblasts displayed nuclear envelope aberrations, presence of prelamin A, proliferation, and senescence rate defects. Gene transcriptional pattern was found differentially modulated between transgenic and wildtype animals, too. In conclusion, the presence of 527His Prelamin A accumulation is further linked to the appearance of mild progeroid features and metabolic disorder without lifespan reduction.

Key words: mandibuloacral dysplasia type A, p.Arg527His pathogenic variant, transgenic mouse model, prelamin A
Introduction

Lamins A/C are major components of the nuclear lamina, playing a fundamental role in the maintenance of the size and shape of the nucleus and in several nuclear processes such as transcription, chromatin organization and DNA replication. Lamins are encoded by LMNA gene, located on 1q.21.1 chromosome region. Pathogenic variants in LMNA gene cause a group of heterogeneous genetic disorders, called laminopathies ranging from muscle-skeletal, cardiac, and peripheral nervous diseases to progeroid diseases. In particular, homozygous or compound heterozygous variants in LMNA gene have been associated to the first identified progeroid laminopathy, known as Mandibuloacral dysplasia type A (MADA; OMIM #248370). This rare autosomal recessive disorder is characterized by the development of mild growth retardation, craniofacial anomalies with mandibular hypoplasia and prominent appearance of the eyes, generalized osteoporosis, osteolysis of terminal phalanges and clavicles, overcrowded teeth and delayed closure of cranial suture usually from the first decade of life. Patients present with lipodystrophy pattern type A, characterized by loss of subcutaneous fat in the extremities and normal or heightened presence of fatty tissue in the neck and trunk. These clinical features are often accompanied by metabolic syndrome including insulin resistance, impaired glucose tolerance and diabetes. In the second decade of life, mild progeroid features become visible, such as thin and sparse nose, thin hair (in both sexes; alopecia is described in males, but is generally less evident e less precocious compared to other progeroid syndromes); the skin appears thin, wrinkled and atrophic over the acral region, with visible veins, and with patchy brown hyperpigmentation area (Acanthosis nigricans). Skeletal and cardiac muscle are not affected in most MADA patients. However, muscle weakness overlapping with other laminopathies have been described in few patients.

The most common causative pathogenic variant of MADA disease is the homozygous transition c.1580G>A, mapping in the exon 9 of LMNA gene, which changes Arginine 527 in Histidine (p.Arg527His) in the C-terminal domain of lamins A/C. This domain presents a carboxyterminal CAAX (cysteine-aliphatic-aliphatic-any aminoacid) motif involved in a complex post-translational processing to produce mature lamin A from the precursor protein, prelamin A. After farnesylation of the cysteine residue, CAAX cleavage, and cysteine methylation, prelamin A undergoes a second proteolytic cleavage removing an additional 15 C-terminal amino acids, producing the mature lamin A protein. Pathogenic variants at C-terminus of lamins A/C, as in MADA, cause an accumulation in the nucleus of prelamin A having deleterious consequences on many cells and tissues, and disease severity is often related to prelamin A abundance. Thus, the first pathogenic event in MADA is the toxic accumulation of mutated prelamin A, provoking abnormal nucleus morphology and a disruption of nuclear envelope organization as demonstrated by anomalous distribution of emerin, SUN1 e SUN2, main nucleoskeleton component, shown in cultured fibroblasts from affected individuals. Moreover, accumulation of prelamin A has deleterious consequences on cellular differentiation in specific tissues, explaining thus some MADA clinical features. Noteworthy, lipodystrophy can be explained by impaired preadipocytes differentiation; in fact, accumulation of prelamin A in these cells can provoke a reduction of SREBP1, the adipocytes transcription factor, due to the binding of prelamin A to SREBP1 and its subsequent admission in the nuclear rim; retention of SREBP1 causes the down-regulation of PPARγ expression reducing thus the rate of preadipocytes differentiation.

Accumulation of prelamin A is involved in impairment of bone tissue turnover causing an excessive production of TGF-β2 levels, a cytokine acting on monocytes to commit them to osteoclastogenesis, from osteoblasts. Increased TGF-β2 levels trigger elevated secretion of osteoprogerin (OPG) and cathepsin K, activating a non-canonical pathway of osteoclast differentiation and increasing resorption activity. Moreover, previous studies found elevated serum levels of matrix metalloproteinase 9 (MMP-9) in MADA patients; such evidence suggests a role of this enzyme in the regulation of bone remodeling, bone resorption and cartilage damage. In addition, accelerated aging in MADA resembles cellular aspects of physiological aging, as nuclear enlargement, and heterochromatin loss. Prelamin accumulation is a trigger of chromatin reorganization, likely mediated by different anchorage or activity of epigenetic factors in the presence of diverse levels of prelamin A. Thus, epigenetic enzymes, such as HDAC2 or SIRT1, are affected in MADA cells, and an increased solubility of heterochromatin protein 1 beta (HP1β) is observed, causing increased histone H4K16 and H3K9 acetylation and decreased H3K9 trimethylation, all age-associated epigenetic marks. Moreover, similarly to other progeroid disorders, MADA cells expressing p.Arg527His show endogenous DNA damage, genomic instability and persistence of unrepaired damage DNA features, probably caused by prelamin A accumulation and, consequently, impaired recruitment of DNA repair protein to the DNA lesion.

The complexity of phenotype in MADA disease could be also explained by a tissue-specific gene expression pattern. MADA fibroblasts present specific up- and downregulation of expression of genes involved in many cellular processes, such as lipid metabolism, cell cycle...
checkpoint, cell adhesion, electron transport and transcription. These data could confirm the main role of lamin A in nuclear transit of transcription factors and, consequently, in transcriptional regulation.

To further provide insights about the consequences of p.Arg527His on the prelamin A accumulation affecting mechanical integrity of the nucleus as well as signaling pathways, we generated a transgenic mouse line overexpressing the most frequent human MADA mutation in LMNA gene in order to understand its contribution to the pathogenesis of the disease.

Materials and methods

Construct of 527His LMNA plasmid

Human LMNA coding sequence (NCBI RefSeq NM_170707) containing the homozygous c.1580G>A substitution has been amplified from mRNA obtained by a MADA patient using the following primers pairs containing the BamH1 and EcoRI restriction sites: R527H-Fw GGATCCATGGAGACCGCTCCGCCAG and R527H-Rv CTTAGTACATGATCTGGCATCTTC. After Sanger sequencing analysis, mutated human LMNA cDNA was inserted between BamH1 and EcoRI restriction sites of the pcDNA3.1 expression vector (ThermoFisher Scientific, Waltham, MA USA) (Fig. 1A).

Generation of 527His LMNA transgenic mice

Transgenic mice were generated at SEAT 44 CNRS Transgenic Mouse Facility at Villejuif (France). PvuI was used to linearize the recombinant 527His LMNA plasmid. Six to seven-week-old C57BL/6J female mice were superovulated by pregnant mare serum gonadotropin and human chorionic gonadotropin. Females were

Figure 1. Generation of 527H LMNA transgenic mice. Schematic representation of gene construct (A) containing a CMV promoter followed by full length cDNA encoding 527His prelamin A, BamH1 and EcoRI restriction endonuclease sites are indicated. The 7.4 kb fragment generated by digestion with PvuI was used for microinjection of pronuclei of fertilized mouse oocytes. 527H LMNA gene expression analysis (B) from three F1 transgenic mice, compared to WT littermates. Sanger sequencing analysis (C) shows the c.1580G>A substitution (arrow).
firstly placed with males for mating, and then sacrificed in the following morning. The ovum was taken for microinjection with the depurated recombinant plasmid. On the next day, the fertilized ovum was put back into the oviduct of pseudopregnant female mice. Newborn mice were obtained after 20 days. Founder transgenic positive mice carrying human c.1580A LMNA gene were identified by PCR and Sanger sequencing analysis and crossed with WT C57BL/6J mice to generate F1 mice for breeding. Phenotypic analyses were performed in parallel with age – and sex – matched litters. All mice were genotyped 2 weeks after birth, amplifying genomic DNA (gDNA) with specific human LMNA primers ghLMNA F GTGAGTGGCAGGGCGTTGG and ghLMNA R GCATCTTTTGTTTCCTACTGGG. Animals were housed according to their gender after weaning in a light – and temperature -controlled facility (12-h light/12-h dark cycle, 21 degree), and allowed free access to food and water. General phenotype characterization of mice (body weight and lifespan) was carried out weekly.

**Copy number assay**

For copy number determination assays of 527His LMNA transgene, we used gDNA isolated from the murine tail. We used TaqMan™ Copy Number Reference Assay RNase P as the standard reference assay for copy number analysis, following manufacturer’s instructions (Applied Biosystems).

**Human LMNA gene expression analysis**

Total mRNA from 527His LMNA fibroblasts was extracted and purified using TRizol reagent (Invitrogen). 527His LMNA cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and gene expression was evaluated by RT-PCR using the following human specific primers: hLMNA F GAGATGATCCTTGCTGACTTAC and hLMNA R GCATCTTTTGGTTTGCCTACTGGG. Animals were housed according to their gender after weaning in a light – and temperature -controlled facility (12-h light/12-h dark cycle, 21 degree), and allowed free access to food and water. General phenotype characterization of mice (body weight and lifespan) was carried out weekly.

**Mice tissue collection and histological analysis**

Animals were euthanized and all in vivo studies were carried out in accordance with European Economic Community Council Directive 86/609, OJ L 358, 1, Dec.18, 1986 and with the NIH-used Guide for the Care and Use of Laboratory Animals 20.

For histological analysis, cutaneous biopsies obtained from the dorsal skin was collected and fixed in 4% paraformaldehyde overnight and embedded in paraffin blocks. The blocks were sectioned into 5 µm-thick slices and place on slides. Hematoxylin and eosin (H&E) staining were performed according to standard protocols. White adipose tissue (WAT) and liver samples were obtained from 4 months old mice, specimens were fixed in 10% paraformaldehyde, and embedded in paraffin. 10 mm consecutive WAT sections were then mounted on slides and stained with H&E. Formalin-fixed liver tissue was processed, and 5-µm-thick paraffin sections were stained with H&E for histological analysis.

**Metabolic assays**

Metabolic testing procedures have been previously described 21,22. Briefly, for oral glucose tolerance tests (OGTT) animals were fasted for 16 hours and injected with 2 g/kg body weight of glucose into the peritoneal cavity; insulin tolerance tests (ITT) were performed by injection of 0.75 units/kg body weight of human regular insulin (Novo Nordisk) into the peritoneal cavity of animals fasted for 6 hours. Blood Glucose concentrations were determined by using an automated Onetouch Lifescan Glucometer. Insulin levels were measured using a commercial kit (Mercodia). Cholesterol and Triglycerides were measured using a Roche Modular T analyzer. Mice were fed a High Fat Diet (HFD, 60% of calories from fat, code D12492 from Research Diets, NY) or standard chow (SC, 10% calories from fat, code 4RF18 GLP Mucedola, Italy) for 20 weeks after weaning as indicated. Studies were performed only in male mice.

**Cell culture**

WT and 527His LMNA fibroblast cultures had been obtained from skin biopsies, using standard procedure. Immediately after collection, the sample was rendered sterile by 3 consecutive washes in PBS (DPBS-Dulbecco’s Phosphate-Buffered Saline; w/o calcium, w/o magnesium; Thermo Fisher Scientific) and antibiotic-antifungal (PAA, The Cell Culture Company), then it was placed in a solution of Dispase (2 mg/mL; Gibco) all night at 4°C, in order to clivate the components of the extracellular matrix. The following day, pieces were incubated with Collagenase I for 4h at 37°C and then they were transferred into Tissue Culture Plates pre-treated with gelatin, in DMEM High Glucose (Gibco) media, containing 10% FBS (Gibco), 1% L-Glutamine (PAA, The Cell Culture Company), 1% Penicillin / Streptomycin (PAA, The Cell Culture Company), 1% NEAA (Gibco) and 0.1% β-mercaptoethanol (Gibco) in a 5% CO2 humidified atmosphere at 37°C.

**Immunofluorescence staining and imaging analysis**

WT and 527His LMNA fibroblasts were fixed and incubated with the appropriate primary antibodies against Prelamin A (C-20; 1:100, Santa Cruz Biotechnology, INC) and Lamin A/C (N-18; 1:100, Santa Cruz Biotechnology, INC). Appropriate Alexa Fluor 488- or 568-la-
beled secondary antibodies were incubated for 1 h at room temperature (Invitrogen, Carlsbad, CA, USA). Cellular senescence was performed using a SA-β-gal staining kit (Cell Signaling, #9860) according to the manufacturer’s instructions. For the proliferation assay, cells were grown on glass coverslips and cultured 24 h. BrdU was added at a concentration of 10 μM for the last 6h after which proliferation assay was performed according to the manufacturer’s instructions (Roche Applied Science). Cell nuclei were labeled with 4,6-Diamidino-2-phenylindole (DAPI-Sigma Aldrich). Images are acquired using a Zeiss (Zeiss, Thornwood, NY, USA) Axioplan 2 microscope. Immunofluorescence analyses were conducted from cellular passage 11 to 17.

**Western blot**

Whole-cell extracts were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). After incubation with 5% milk in TBST (10 mM Tris, 150 mM NaCl, 0.5% Tween 20 [pH 8.0]) for 1 hour, the membrane was incubated with indicated antibodies overnight at 4°C. Membranes were washed with TBST three times and incubated with a 1:5,000 dilution of horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibodies for 1 hour. Blots were washed with TBST three times and developed with the ECL system (Bio-Rad).

**Microarray analysis and processing**

Total RNA from dermal fibroblasts derived from adult (1 year) mice overexpressing 527His LMNA was extracted and purified using TRIZol reagent (Invitrogen); its quality and quantity was assessed using a Nanodrop spectrophotometer (Thermo Scientific) and agarose gel electrophoresis. Synthesis of the labelled first strand cDNA was conducted according to manufacturer’s instructions (One-Color Microarray-Based Gene Expression Analysis, Agilent) with starting material of 1 μg of total RNA. The labeled cDNAs were co-hybridized to the Whole Mouse Genome Oligo Microarray (G4122A, Agilent) in duplicate, with one dye swap. Whole Mouse Genome Oligo Microarray Kit slides contained about 44,290 oligonucleotides corresponding to 41,174 genes and transcript (www.agilent.com/chem/dna). Detailed methods for sample processing and microarray experiments have been previously described 23. Image analysis and processing were performed as described in Tiano et al., 2020 24.

**Validation of relative gene expression by real-time RT-PCR**

1 μg of total murine RNA has been retrotranscribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to evaluate the expression levels of selected differentially expressed genes (DEGs). We analysed the following genes: Igf2, Fgf110, Epyc, Zic1, Fst, Pdgfc, Tnfrsf21, and Chi3l1. Real time PCR (qRT-PCR) has been performed using ABI7500 Fast Real-time PCR System (Life Technologies) and murine Taqman assays (Applied Biosystems, USA). All samples were run in triplicate and average values were calculated. Two independent reverse transcriptions were tested for each gene. Relative quantification of gene expression among each sample was achieved by normalization against Gapdh as endogenous control using the ΔΔCt method of quantification 23.

**Functional analysis and pathway enrichment analysis**

We used KEGG pathway enrichment analysis for the DEGs analysis. KEGG pathway enrichment analysis for the DEGs is a comprehensive database resource, which consists of chemical information, genomic information and systems information (REF). Enrichment analysis of KEGG pathways of DEGs was done by “clusterProfiler” R package to explore the most likely gene function 25. P < 0.05 was used as the cut-off criterion.

**Statistical analysis**

All data were expressed as means ± SD. For in vivo studies, three to six mice per genotype per assay were used. For in vitro cell studies, each experiment was repeated at least three times. Data were analyzed by Student t test, two-way ANOVA, and post hoc test (GraphPad Prism 8). The significance level was set at p < 0.05 (*p < 0.05, **p < 0.01).

**Results**

**Generation of 527His LMNA transgenic mouse and disease phenotype**

The general structure of the 527His LMNA gene construct is shown in Figure 1A. 527His LMNA gene expression assessment and Sanger sequencing analysis were determined in studied F1 generation mice (Fig. 1B-C), after copy number assay (data not shown). Transgenic progeny of F1 and subsequent generations was born at the expected ratio of approximately 1:1 when compared with nontransgenic littermates. At birth, the macroscopic appearance of MADA transgenic mice was indistinguishable from their WT siblings. All mice were lively, active, explorative, and eating, drinking and interacting with cage mates. By the 2nd month of age, most animals were smaller than wild type littermates (Fig. 2A). 527His LMNA mice grow slightly less and gain weight slowly than their littermates until 30th week of age for the males and until 44th week of age for the females (Fig. 2B). At fifth month, we observed loss of hair in transgenic animals, compared with
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WT mice (Fig. 2C). Kaplan-Meier survival curves did not reveal any differences in the lifespan (Fig. 2D).

**Metabolism**

Glucose tolerance and insulin sensitivity were comparable in WT and 527His LMNA mice fed regular chow for 12 weeks (not shown) or 24 weeks (Fig. 3A-B). When mice were challenged with a diet rich in fat (HFD) we did not observe differences in body weight (Fig. 3C). Moreover, 527His LMNA animals revealed no differences in serum lipids levels (Fig. 3D), a mild significant glucose intolerance and insulin resistance (Fig. 3A-B) and slightly higher insulin levels in the fed state (Fig. 3E). Histological analysis of tissues relevant for metabolic homeostasis such as white adipose tissue and liver revealed no gross differences between WT (Fig. 4A,C) and 527His LMNA (Fig. 4B,D) littermates apart mild increase in inflammatory cells in adipose tissue from transgenic animals.

**Skin**

Microscopic analysis of dorsal skin sections obtained by cutaneous biopsy from transgenic mice at 24 weeks of age, showed a reduction of hypodermal thickness compared with WT littermates (Fig. 4E,F), due to the loss of subcutaneous adipose tissue.

**Cellular results**

Once established primary cell lines from murine dermal fibroblasts, cells were morphologically characterized in vitro. Irregularly shaped nuclei with intra/transnuclear membrane invaginations, large protrusions (“buds” or “blebs”) or doughnut-shaped nuclei, and independent nucleus-like structures, were detected in 20% of all MADA cells and in 12% of WT ones (Fig. 5A,B, p-value < 0.05). In order to analyze the nuclear envelope, immunofluorescence analyses were conducted both for the detection of prelamin A and mature lamin A. Prelamin A showed an abnormal accumulation in about 66% 527His LMNA nu-
clei mostly located at the nuclear rim, within membrane invaginations and occasionally in intranuclear structures (Fig. 5A). As expected, prelamin A rarely detected (9%) in WT cells (p-value < 0.01) which don’t show any nuclear alterations (Fig. 5A). Meanwhile, lamin A is expressed in all nuclei both in WT and 527His LMNA fibroblasts with the same rim nuclear distribution (Fig. 5B). The data have been reported in a histogram (Fig. 5C). We also performed a Western blot analysis showing the presence of Prelamin A in primary cellular lines from two different 527His LMNA mice while no appreciable signal was visible in WT. Hela cells accumulating Prelamin A after treatment with Farnesyltransferase inhibitors (FTI) were used as control (Fig. 5D).

In addition, 527His LMNA fibroblasts proliferated at lower rate than WT as underlined by BrdU assay especially at high culture passages, (i.e 15 and 17) (Fig. 5E), (p < 0.01). Moreover, under normal growth conditions, 527His cells showed an increased percentage of senescence associated β-galactosidase staining especially at passage 13 (12% in WT and 34% in 527His LMNA cells) (Fig. 5F, p-value < 0.05).

**Analysis of differentially expressed genes (DEGs)**

After filtering out unreliable probe sets with expression at background level, 222 out of 41,174 murine genes and transcript were considered as significant expressed in adult fibroblasts derived from mice overexpressing 527His

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**Figure 3.** Metabolic assessment of WT vs 527His LMNA mice at regular chow and high fat diets (20 weeks). Measurement of glucose tolerance (A) and insulin sensitivity (B) in both diet conditions. No differences were observed in body weight (C) and serum lipids (D) during the HFD diet. Slight higher insulin levels (E) were showed for 527His LMNA vs WT mice in the fed state.
Figure 4. Representative images of histological analysis of white adipose tissue, liver and skin tissues in WT (A, C, E) vs 527His LMNA mice (B, D, F). Mild increase in adipose tissue inflammatory cells was noted in 527His LMNA mice. No differences were observed in liver. Reduction of hypodermal thickness was viewable in 527His LMNA mice. Haematoxylin and eosin (H&E) staining.
Figure 5. Representative image of immunofluorescence staining showing the abnormal presence of prelamin A (A) in 527His LMNA nuclei and aberrant nuclear envelope conformations, while control cells (WT) show regular nuclear envelope shape and a rarely detection of prelamin A staining. Meanwhile, lamin A is expressed in all nuclei both in WT and 527His-dermal fibroblasts with the same rim nuclear distribution (B). DAPI nuclear staining (blue). Scale bar 100 µm. Histogram (C) represents the percentage of aberrant nuclear conformations. Prelamin A and lamin A positive nuclei. Error bars represent the SD from the analysis of 100 cells from three independent experiments and WT values are displayed as the average percentages of 2 different controls (**p < 0.01). Representative Western blot analysis (D) of Prelamin A, Lamin A and C of equal amount of total proteins from 527His LMNA (n = 4) and WT mice fibroblasts (n = 5). Protein extracts of Hela cells treated with FTI were used as positive control. β-actin was used as control. Data are presented as means ± SD. Representative immunofluorescence images (E) showing the presence of BrdU positive cells in 527His and WT mice cells. DAPI nuclear staining (blue). Scale bar 100µm. The histogram shows the percentages related to BrdU positive cells in 527His cells at passages 11,13,15,17. Error bars represent the SD from the analysis of 100 cells from three independent experiments. WT and MADA values are displayed as the average percentages of 5 different mice respectively (*p < 0.05, **p < 0.01). Representative image (F) of senescence-associated β-galactosidase assay at passage 13. A greater amount of intensely positive blue cells are displayed in 527His-fibroblasts than WT controls. The histogram shows the average percentage of β-galactosidase-positive cells in WT and 527His LMNA fibroblasts at passage 11,13,17. Error bars represent the SD from the analysis of 100 cells from three independent experiments. WT and MADA values are displayed as the average percentages of 5 different mice respectively (*p < 0.05).
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LMNA. Twenty-nine up-regulated and 37 down-regulated transcripts out of 66 DEGs were detected according to the criteria of “Benjamini adjusted p-value < 0.05” and “FC ≥ ± 1.5” (Tab. I). The expression levels of 11 DEGs were confirmed by QRT-PCR analysis.

Pathway analysis

KEGG pathway analysis identified the main molecular pathways altered in adult fibroblasts derived from mice overexpressing 527His LMNA (Tab. II). KEGG analysis recognizes that the 66 DEGs were significantly enriched in multiple pathways including cell signaling, pathways in cancer, immune system, human diseases. The pathway “Environmental Information Processing” that comprehends membrane transport, signal transduction and signaling molecules and interaction pathways, resulted the most significant enriched pathway in mutant fibroblasts (p < 0.005; Tab. II). Noteworthy, inside this general pathway, “Signaling molecules and interaction” pathway was significant enriched too (p < 0.0001, Tabs. II-III). Nine DEGs belongs to this pathway: Il6,

Table I. DEGs (FC≥ ± 1.5) in adult mice fibroblasts.

| Gene name  | Accession number | FC  | P-value        | Gene position |
|------------|------------------|-----|----------------|---------------|
| 1 Pdlim3   | NM_016798        | 8.63| 1.13E-07       | chr8          |
| 2 Onecut2  | NM_194268        | 3.45| 1.87E-07       | chr18         |
| 3 Epyc     | NM_007884        | 3.08| 5.59E-06       | chr10         |
| 4 Tmef2    | NM_019790        | 2.86| 9.12E-07       | chr1          |
| 5 Agtr1a   | NM_177322        | 2.41| 4.53E-05       | chr13         |
| 6 Zic1     | NM_009573        | 2.41| 0.000150879    | chr9          |
| 7 Gria4    | NM_019691        | 2.29| 3.89E-06       | chr9          |
| 8 Cpz      | NM_153107        | 2.21| 7.14E-05       | chr5          |
| 9 Spon2    | NM_133903        | 2.16| 2.33E-05       | chr5          |
| 10 Fndc5   | NM_027402        | 2.13| 0.000158579    | chr4          |
| 11 I1r2    | NM_010555        | 1.97| 1.14E-05       | chr1          |
| 12 Rpl39l  | NM_026594        | 1.91| 2.81E-05       | chr16         |
| 13 Osr1    | NM_011859        | 1.89| 6.07E-05       | chr12         |
| 14 Trib3   | NM_175093        | 1.86| 0.00023456     | chr2          |
| 15 Olfl2b  | NM_177068        | 1.85| 9.26E-06       | chr1          |
| 16 Aldh1/2 | NM_153543        | 1.79| 0.000183763    | chr10         |
| 17 Angpt2  | NM_011923        | 1.75| 0.000169423    | chr2          |
| 18 Lama2   | U12147           | 1.72| 1.03E-19       | chr10         |
| 19 Emilin2 | NM_145158        | 1.69| 0.000202906    | chr17         |
| 20 2210409E12Rik | AK008869     | 1.68| 5.07E-05       | chr11         |
| 21 Tnfrsf21| NM_178589        | 1.60| 0.000112189    | chr17         |
| 22 Lpl     | NM_008509        | 1.60| 0.000169284    | chr8          |
| 23 C2      | NM_013484        | 1.59| 0.00031931     | chr17         |
| 24 I1r5a   | NM_008358        | 1.57| 0.000182628    | chr2          |
| 25 Sh3bp5  | NM_011894        | 1.56| 0.000201258    | chr14         |
| 26 D0H4S114| NM_053078        | 1.56| 0.00012852     | chr18         |
| 27 Moeo2   | NM_008584        | 1.56| 3.20E-06       | chr12         |
| 28 S1pr3   | NM_010101        | 1.55| 0.000151178    | chr13         |
| 29 Cebpa   | NM_007678        | 1.51| 4.19E-05       | chr7          |
| 30 Cd28    | NM_007642        | -1.52| 9.62E-13       | chr1          |
| 31 Tspan6  | NM_019656        | -1.53| 0.000171921    | chrX          |
| 32 Lxn     | NM_016753        | -1.54| 0.00013059     | chr3          |
| 33 Saa1    | NM_009117        | -1.54| 0.000289162    | chr7          |
| 34 Kctd12  | NM_177715        | -1.55| 0.00015173     | chr14         |
| 35 Ccbe1   | NM_178793        | -1.57| 3.78E-05       | chr18         |
| 36 Fgf10   | NM_008002        | -1.57| 3.39E-15       | chr13         |
| 37 Tnfsf11 | NM_011613        | -1.60| 3.95E-05       | chr14         |
Tnfsf11, Pdgfc, Pdgfa, Il1r2, Il15ra, Tnfrsf21, Lama2, S1pr3 (Tab. III). Seven genes out of nine are in “Cytokine-cytokine receptor interaction” (mmu04060) pathway (Tab. III).

In addition, the pathway named “Human diseases” showed a significant enrichment (p < 0.05, Tab. II). Inside this large pathway, two specific pathways are significant enriched in mutant fibroblast: Cancer (p < 0.005) and Neurodegenerative diseases (p < 0.05). Five DEGs are part of “Cancer” pathway, while three DEGs of “Neurodegenerative diseases” (Tab. III). Finally, also the “Immune system” pathway revealed significant enriched (p < 0.05) with five DEGs (Tab. III).

**Discussion**

Studies during the past 15 years have established that several progeroid syndromes are caused by genetic defects that interfere with the processing of prelamin A to mature lamin A. It is known that the balance between these two proteins triggers the severity of ageing.

MADA is caused by point mutations in C-terminal domain of LMNA gene that through an unknown process produce a mutated prelamin A. MADA patients show a mild accelerate aging compared to patients with Hutchinson-Gilford progeria syndrome (HGPS), caused by the presence of truncated prelamin A (progerin), that lacks the endoproteolytic cleavage site domain that would normally release mature lamin A. The absence of mature lamin A causes a more severe progeroid disorder, restrictive dermopathy (RD), caused by homozygous loss of ZMPSTE24 enzyme involved in the cleavage of C-terminal of prelamin A. Partial loss of ZMPSTE24 activity has been associated Mandibulocral Dysplasia type B (MADB) with severe metabolic syndrome, abnormal fat accumulation and dilated cardiomyopathy 26-28.

The toxic accumulation of mutated prelamin A provokes alterations of nuclear morphology, perturbations of cell cycle, defects in cellular replication, senescence rate,
| Pathway                                      | Genes on slides/pathway | DEG/pathway | P-value      |
|----------------------------------------------|-------------------------|-------------|--------------|
| Signaling molecules and interaction         | 683                     | 9           | 0.000035     |
| Cancers                                     | 335                     | 5           | 0.002        |
| Environmental information processing        | 1472                    | 10          | 0.0029       |
| Human diseases                              | 1054                    | 7           | 0.02         |
| Immune system                               | 714                     | 5           | 0.045        |
| Neurodegenerative diseases                  | 287                     | 3           | 0.046        |
| Cellular processes                          | 1101                    | 4           | n.s.         |
| Genetic information processing              | 1094                    | 1           | n.s.         |
| Metabolism                                  | 1358                    | 2           | n.s.         |
| Organismal systems                          | 2421                    | 7           | n.s.         |
| Cardiovascular diseases                     | 187                     | 2           | n.s.         |
| Cell communication                          | 391                     | 3           | n.s.         |
| Cell growth and death                       | 291                     | 1           | n.s.         |
| Cell motility                               | 198                     | 2           | n.s.         |
| Development                                 | 180                     | 1           | n.s.         |
| Endocrine system                            | 433                     | 2           | n.s.         |
| Glycan biosynthesis and metabolism          | 202                     | 1           | n.s.         |
| Immune system diseases                      | 185                     | 1           | n.s.         |
| Infectious diseases                         | 270                     | 1           | n.s.         |
| Lipid metabolism                            | 335                     | 1           | n.s.         |
| Signal transduction                         | 961                     | 5           | n.s.         |
| Translation                                 | 431                     | 1           | n.s.         |

n.s.: not significative

| Gene name | Accession number | FC      | P-value       | Pathway                                      | C1                          | C2                          | C3                          |
|-----------|------------------|---------|---------------|----------------------------------------------|-----------------------------|-----------------------------|-----------------------------|
| Fst       | NM_008046        | -1.86   | 5.64E-05      | mmu04350 Environmental information processing | Signal transduction         | TGF-beta signaling pathway  |
| II15ra    | NM_008358        | 1.57    | 0.0002        | mmu04060 Environmental information processing | Signaling molecules and interaction | Cytokine-cytokine receptor interaction |
| II15ra    | NM_008358        | 1.57    | 0.0002        | mmu04630 Environmental information processing | Signal transduction         | Jack/STAT signalling pathway |
| II15ra    | NM_008358        | 1.57    | 0.000183      | mmu04672 Organismal system                   | Immune system               | Intestinal immune network for IgA production |
| II1r2     | NM_010555        | 1.97    | 1.14E-05      | mmu04060 Environmental information processing | Signaling molecules and interaction | Cytokine-cytokine receptor interaction |
| II1r2     | NM_010555        | 1.97    | 1.14E-05      | mmu04010 Environmental information processing | Signal transduction         | MAPK signalling pathway     |
| II6       | NM_031168        | -1.64   | 1.11E-14      | mmu04060 Environmental information processing | Signaling molecules and interaction | Cytokine-cytokine receptor interaction |
| II6       | NM_031168        | -1.64   | 1.11E-14      | mmu04630 Environmental information processing | Signal transduction         | Jack/STAT signalling pathway |
| Gene name | Accession number | FC   | P-value   | Pathway                                      | C1                         | C2                                 | C3                                      |
|----------|------------------|------|-----------|----------------------------------------------|----------------------------|------------------------------------|-----------------------------------------|
| Il6      | NM_031168        | -1.64| 1.11E-14  | mmu05200 Human disease                       | Cancers                   | Pathways in cancer                  |                                         |
| Il6      | NM_031168        | -1.64| 1.11E-14  | mmu05020 Human disease Neurodegenerative     | Diseases                  | Prion diseases                      |                                         |
| Il6      | NM_031168        | -1.64| 1.11E-14  | mmu04623 Organismal system                   | Immune system             |                                    | Cytosolic DNA-sensing pathway; hemat-   |
|          |                  |      |           |                                              |                            |                                    | apoietic cell lineage; intestinal      |
|          |                  |      |           |                                              |                            |                                    | immune network for IgA production;     |
|          |                  |      |           |                                              |                            |                                    | NOD-like receptor signaling pathway;   |
|          |                  |      |           |                                              |                            |                                    | toll-like receptor signaling pathway;  |
|          |                  |      |           |                                              |                            |                                    |                                         |
| Lama2    | U12147           | 1.72 | 1.03E-19  | mmu04512 Environmental information processing | Signaling molecules and   | ECM-receptor interaction             |                                         |
|          |                  |      |           |                                              | interaction               |                                    |                                         |
| Lama2    | U12147           | 1.72 | 1.03E-19  | mmu05020 Human disease                       | Cancers                   |                                    | Small cell lung cancer; Pathways in    |
|          |                  |      |           |                                              |                            |                                    | cancer                                  |
| Pdgfa    | NM_008808        | -1.70| 8.61E-06  | mmu04060 Environmental information processing | Signaling Molecules and   |                                    | Cytokine-cytokine receptor interaction |
|          |                  |      |           |                                              | Interaction               |                                    |                                         |
| Pdgfa    | NM_008808        | -1.70| 8.61E-06  | mmu04010 Environmental information processing | Signal transduction       |                                    | MAPK signalling pathway                |
|          |                  |      |           |                                              |                            |                                    |                                         |
| Pdgfc    | NM_019971        | -2.28| 4.21E-06  | mmu04060 Environmental information processing | Signaling Molecules and   |                                    | Cytokine-cytokine receptor interaction |
|          |                  |      |           |                                              | Interaction               |                                    |                                         |
| Pdgfc    | NM_019971        | -2.28| 4.21E-06  | mmu05218 Human disease                       | Cancers                   |                                    | Melanoma; prostate cancer              |
|          |                  |      |           |                                              |                            |                                    |                                         |
| S1pr3    | NM_010101        | 1.55 | 0.00015   | mmu04080 Environmental information processing | Signaling molecules and   |                                    | Neuroactive ligand-receptor interaction |
|          |                  |      |           |                                              | interaction               |                                    |                                         |
| Tnfrsf21 | NM_178589        | 1.60 | 0.0001    | mmu04060 Environmental information processing | Signaling molecules and   |                                    | Cytokine-cytokine receptor interaction |
|          |                  |      |           |                                              | interaction               |                                    |                                         |
| Tnfsf11  | NM_011613        | -1.60| 3.95E-05  | mmu04060 Environmental information processing | Signaling molecules and   |                                    | Cytokine-cytokine receptor interaction |
|          |                  |      |           |                                              | interaction               |                                    |                                         |
| Cebpa    | NM_007678        | 1.51 | 4.19E-05  | mmu05221 Human disease                       | Cancers                   |                                    | Acute myeloid leukemia, Pathways in    |
|          |                  |      |           |                                              |                            |                                    | cancer                                  |
527His prelamin A accumulation in MADA mouse model

and changes of chromatin organization affecting gene transcriptional processes. In particular, these effects on nuclear dynamics may account for many of the clinical features and tissue-specific alterations observed in human progeroid laminopathies. The characterization of 527His LMNA transgenic mice confirms and extends these evidences. The transgenic MADA mice generated overexpressing 527His prelamin A showed a significative percentage of nuclei with morphological alterations of envelope shape and a reduction of cellular proliferation with an increase of senescence rate.

According to numerous studies in MADA and HGPS animal and cellular models supporting that accumulation of prelamin A affects changes in gene expression levels, we explored the transcriptional pattern in 527His LMNA transgenic fibroblasts compared to WT cells. Sixty-six DEGs are implicated in distinct pathways. The most significant enriched pathways in mutant fibroblasts comprehend signal transduction, cytokine-cytokine and extracellular matrix (ECM)-receptor interaction pathways. These changes are reminiscent of the effect of the Senescence-associated Secretory phenotype and suggest that MADA effects on gene expression might affect tissue integrity or regeneration via systemic inflammation. In fact, in experimental models overexpressing 527His LMNA mutation or in MADA patients’ serum it has been observed an alteration of cytokine secretion and ECM enzymes release and activity. This aspect has to been further investigated in order to clarify the pathogenic aspect and to develop therapeutic strategies for MADA and other age-related disorders.

Muscle phenotype is apparently normal in our transgenic mice, reflecting the human p.Arg527His MADA phenotype. On the contrary, MADB or RD patients with mutations responsible for a partial or complete abolishment of the catalytic function of the ZMPSTE24 enzyme, develop muscle weakness and cardiovascular disease. Zmpste24 deficiency in murine models determines progeroid features with muscle weakness or cardiomyopathy and muscular dystrophy. These data link high levels of prelamin A with altered structure of the nuclear lamina that could affect mechanically stressed tissues such as the muscle fibers of the heart and skeleton. The relative low levels of prelamin A in 527His LMNA transgenic mouse and MADA patients may be not sufficient to determine a muscle damage.

Notably, in this study, transgenic MADA mice described showed a mild phenotype, with a minimal change in body weight and a normal rate of survival compared with WT animals. Glucose metabolism and insulin sensitivity were comparable in WT and 527His LMNA mice fed regular chow and just a mild significant glucose intolerance and insulin resistance in 527His mice is observed when animals were challenged with a diet rich in fat. Nevertheless, we observed typical cutaneous alterations of MADA patients, such as loss of hair and decrease of subcutaneous adipose tissue. A possible explanation of the observed mild phenotype could be the known correlation between the efficiency levels of prelamin A maturation process and disease severity.

In conclusion, our 527His LMNA transgenic model resembles the mild phenotype observed in individuals with hereditary MADA laminopathy and may provide additional evidence about the role of nuclear integrity, specific biological pathways and transcriptional changes in order to in depth understand the pathological and physiological aging.

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| Gene name | Accession number | FC  | P-value  | Pathway          | C1                | C2                | C3                |
|-----------|------------------|-----|----------|------------------|-------------------|-------------------|-------------------|
| Lpl       | NM_008509        | 1.60| 0.0002   | human disease    | neurodegenerative diseases | Alzheimer’s disease |
| Mme       | NM_008604        | -1.95| 4.46E-05 | human disease    | neurodegenerative diseases | Alzheimer’s disease |
| Mme       | NM_008604        | -1.95| 4.46E-05 | organismal system | immune system     | hematopoietic cell lineage |
| Cfh       | NM_009888        | -1.66| 1.46E-06 | organismal system | immune system     | complement and coagulation cascades |

Table III. continue
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