Final Report Summary - MYOAGE (Understanding and combating human age-related muscle weakness)

Executive Summary:
Ageing is a hot topic due to the changing demography of developed countries that have seen life expectancy reach an all-time high. The percentage of the population made up of people aged over 65 years increased in all European Union (EU) member countries between 1985 and 2011 and is projected to further increase in all EU countries over the next 20 years. The continuing improvement in mortality rates is an achievement to be celebrated, but we must not overlook the problem that most people aged over 65 years report long-standing illness or disability that reduces their quality of life and restricts their ability to be economically or socially active. Among the many comorbidities of ageing, musculoskeletal dysfunctions are the most common. Thus, a major challenge to researchers, government and, in fact, society at large, is
to find ways to preserve health and vitality into older age. In the elderly, muscles become atrophic (loss in muscle mass) and weaker (loss in muscle force), more susceptible to damage and regenerate and recover more slowly than was the case in their youth. Understanding and combating age-related muscle weakness requires a precise definition of the elderly population in terms of mobility and repair capacity, based upon assessment and identification of both physiological and molecular indicators and integration of this data to replace the fragmentated and dispersed knowledge that we have of age related muscle weakness. Ageing is a general process of the organism, in which a decline in the proliferation of the progenitors, a lower income of nutrients through vasculature as well as a decreased efficiency of the cellular machinery to metabolize these nutrients, and a less effective dialogue with the other systems participating to the muscle function, result finally in muscle weakness and frailty. Muscle ageing is thus necessarily a multi-component process which will involve as targets the muscle cells, the inflammatory process that increases with aging, as well as weaker tendons and less effective control by the nerves. Combating muscle weakness requires an integrated multi-disciplinary approach that gathers expertise from gerontologists, epidemiologists, cellular and molecular biologists and physiologists.

The MYOAGE study was an EU-FP7-funded large-scale project involving 19 leading academic or industry-based research teams from 10 EU countries. The project encompassed an integrative approach of epidemiological, physiological, cell and molecular and genetic investigations of patients suffering chronic disease involving muscle weakness, compared to healthy humans and master athletes with developed muscle strength, as well as rodent and cell culture models. The overall aim was to investigate the contribution of age-related changes to muscle mass, contractile characteristics and neural control in relation to mobility limitations in healthy young and older adults using standardised assessments. This multi-disciplinary pan-European approach has allowed us to define healthy and impaired muscle ageing and to identify new biomarkers and risk factors of muscle weakness in older age. The project was organised in three main aims: Collect, collate and combat. These aims were achieved through eight work packages (WPs) with a scientific focus, 1 WP specifically addressing ethical issues and 2 WPs dealing with overall study management and training of emerging scientists. MYOAGE reported the detailed methodology and phenotype data collected from 504 young and older healthy men and women. Biological material (muscle biopsies, muscle cell cultures, blood and serum samples) were collected from these well characterised young and old sedentary and active subjects and was used by all research groups to try to correlate the age related physiological modifications to cellular and molecular mechanisms.

One striking theme to emerge from the integration of results from this project was that findings from animal models are not necessarily an indication of human ageing. Detailed examination of human tissue offered several “surprise” findings and emphasized the role of an “ageing environment” on muscle function and regenerative capacity. However, there was considerable “heterogeneity” between samples that often made it difficult to reach final conclusions about the respective role of the molecular processes in healthy or diseased ageing. This combined effort should be followed up in larger human trials.

The MYOAGE ethos was to promote multi-disciplinary, collaborative work between established scientists and industry as well as the development and mentorship of emerging young scientists. The results of this work provide a detailed insight into “normal, healthy” ageing as well as disease and link whole-body function and systems-biology to the structure and function of the neuromuscular systems and the molecular characteristics of skeletal muscle. The work will be used to define guidelines for public health promotion in older age. All data have been retained in a database. A biobank including blood and muscle tissue samples is being maintained and we welcome opportunities to collaborate and further develop our understanding of the impact of ageing on neuromuscular function and health. Finally, several workshops
were organized for young students and scientists, thus paving the way to a body of confirmed scientists within the field of muscle ageing.

In conclusion, the concerted research projects conducted within MYOAGE, and which associated clinicians, cell and molecular biologists and physiologists, most of the members having multiple expertise, confirmed some data described on animal models, but also infirmed others, thus designing a new landscape representing human muscle ageing. This standardized description of physiological, cellular and molecular mechanisms involved will allow us to consider ageing of each organ as integrated into the ageing organism in order to identify relevant targets to provide to the EU population conditions for “healthy ageing

Project Context and Objectives:
MYOAGE, a European Commission FP7 funded large collaborative effort to understand and combat age related-muscle weakness
The ageing of the European population is a major public health concern for most of the industrialised western countries, and represents both a social and an economic burden of the European population. An important aspect of ageing is the impact of the ageing on the skeletal muscle, resulting in a progressive loss of mobility and autonomy that decreases the quality of life and in more frequent falls which, combined to a less efficient regenerative capacity, has major economic and social consequences for the society at large. Progressive muscle weakness is a major component of muscle ageing, with occurrence of muscle atrophy (loss in muscle mass, as shown in fig. 1) and weakness (loss in muscle force), more susceptible to damage and consequently regenerate and recover more slowly than was the case in their youth.

MYOAGE, a large scale collaborative project funded by the European Commission under the 7th Framework programme gathers the best experts of muscle ageing in Europe in order to:
• identify the relative importance of muscle weakness in the European population today and propose standards to define healthy ageing;
• identify molecular mechanisms and pathways which are responsible for this weakness and which may be targeted to combat age related muscle weakness
• identify therapeutic strategies to prevent muscle loss and weakness, increase health span and enhance recovery following injury or immobilisation.

Muscle atrophy in ageing: a multifactorial process
The major component of the muscle atrophy is the sarcopenia. Sarcopenia is a universal, age-related, loss of muscle mass associated with a loss of strength and function resulting in muscle weakness. It can start as early as 30 year of age and can result in a loss of about 30-50% of the muscle mass by the age of 80 years. Diagnosis of loss in muscle mass is particularly difficult since there are no standards to define healthy muscle ageing as opposed to debilitating muscle weakness. Many factors are involved in the aetiology of muscle weakness and sarcopenia such as denervation, chronic inflammation, hormonal and nutritional changes, and modification in life style.

Figure 1: scheme of the multifactorial process leading to sarcopenia

Since inactivity is itself a cause of muscle wasting this constitutes a vicious circle that is hard to break out of. Although 30-40% muscle loss is an important factor limiting activity, the major challenge occurs as a
result of traumatic events, accident, fracture or illness, that result in immobilisation which leads to a very rapid loss of an additional 20-30% of muscle mass over a six week period. We know that without adequate muscle regeneration after events such as hip fracture, many elderly people become bed-ridden or housebound, and have a high mortality in the year following their accident; very few of those who survive ever regain full mobility. Reducing the initial wasting and speeding up recovery is thus a major target to counteract the decrease in mobility and independence of the elderly population.

MYOAGE: from understanding to combating age-related muscle weakness

MYOAGE is a large scale collaborative project funded by the European Commission under the 7th Framework Programme. Coordinated by Inserm and managed by Inserm-Transfert, it gathers a total of 19 institutions represented by the best experts in Europe to cover all aspects of the field of muscle wasting with age. This will lead to the establishment of a network which will cover a field going from the specialists who will collect clinical and physiological data from elderly persons to the experts able to decipher the finest molecular mechanisms behind the muscle wasting. Altogether we will be able to define recommendations and interventions to combat the impact of the loss of muscle mass (Fig. 2).

Figure 2: illustration of the MYOAGE strategy

Project Results:
MYOAGE has been a highly productive collaborative effort which has led to many advances in the accumulation of evidences on muscle ageing process. Here below are described the results obtained per workpackage.

Work Package 1
MYOAGE defined parameters of healthy versus impaired muscle ageing, and describes possible strategies to combat sarcopenia. This coordinated effort required investigating muscle tissue from well characterised elderly (>70 years of age) individuals with and without muscle weakness, in comparison to tissue from healthy young adults (18-40 years of age) as controls. Since the final aim was to characterize and promote healthy ageing, physiological assessment of the subjects was carried out in parallel to cellular and molecular investigations, in order to clearly define the phenotype and biomarkers of age related muscle weakness. However, the size of the biopsies that can be taken from such subjects is necessarily limited, and these biopsies should be used only for informative investigations. This is why Myoage organized sample collected in two phases. The first phase aimed at optimizing protocols and strategies, using phase I biopsies which were surgical remnants from patients undergoing hip or knee surgery (muscle biopsies from m. vastus lateralis). These biopsies were used to isolate cell cultures. They were also used to assess the biochemical strategies to investigate signalling pathways that could be deregulated during ageing, and which could represent targets to combat sarcopenia. Phase II biopsies were obtained from very well phenotyped individuals from different ages with and without muscle weakness.

The so-called phase I samples have been used by the consortium for the optimisation of all protocols using human biological materials and for all pilot studies on the translation of basic biological data obtained with model organisms / systems to human material. In parallel muscle biopsies (typically 200-500 mg per subject), blood samples (in total 40 ml/subject) were also collected and distributed or bio-banked. Other samples were used for histology, the isolation of mRNA and for the isolation of proteins (listed in the
different WPs). Data and protocols were exchanged and provided extremely useful information. Well characterised phase 1 samples are still being used by the different groups especially those working on age related post-translational protein modifications (WP6). This is due to the fact that phase 2 biopsies proved to be too small (around 50-200mg max) to carry out the biochemical analysis. In Total, 258 patients of a wide range of ages were included in Leiden, and 80 patients were included in Bologna, including oldest old and young individuals (younger than 40 and older than 80 years of age). For these patients, epidemiological data related to the samples were collected. This includes information on lifestyle, comorbidity, use of medication, menopause, anthropometry, activity reporting, living status, falls, quadriceps ultrasonography (Bologna). The results of all analyses are added to a database.

The second phase concerned well characterised subjects as described in WP2. The categories were redefined among the consortium as young subjects, old active subjects and old sedentary subjects, keeping in mind the necessity to equilibrate between males and females. All partners had to carefully adapted extraction procedures due to the small size of the phase II biopsies, an optimization which should profit to all EU scientific community working in this field. Phase II samples were collected by the 5 centers (P01, P01.2 P01.3 P06, P08, P09) and have been redistributed to carry out the various tasks of the WPs.

In Leiden muscle and blood has been collected in a total number of 110 subjects. All muscle biopsies have been distributed to partners for further analysis: 19 (Inserm), 3 (Musaro), 6 (UNIBO), and partner 12 (UPMC). Blood samples have been distributed to partner 14 (Unilever), partner 4 (RHC) and 6 (UNIBO) for analysis. Epidemiological and clinical data as well as all the measures from WP2 have been transmitted to partner 8 LUMC and have been incorporated into an electronic data base. The collection of biological samples in being maintained in Leiden in order that it can be continued to be used to finish ongoing studies over the next few years.

Biopsies from 5 subjects in each group were sent the partner 1a in order to establish muscle cultures from the resident adult stem cell population. These cells were amplified and distributed to the different workpackages and interesting results on signalling mechanisms and protein modifications during aging were found (see WPs).

SOP’s have been defined for sampling procedures, for exclusion criteria, for transportation and for cell culturing techniques. A database has been created by Partner 8 in which all partners entered their data. This will also be continued outside of the framework of MYOAGE.

Work Package 2
Workpackage 2 was a multi-centre, Pan-European collaborative study of healthy ageing in humans. The overall aims were: to complete detailed assessments of muscle changes during healthy ageing; and to determine the contribution of age-related changes to muscle mass, contractile characteristics and neural control to reductions in mobility in older age. In total, 504 healthy participants were examined across five test-centres. All volunteers were independent living, socially active and free from disease that impaired mobility levels. This workpackage was a comprehensive large-scale physiology-based study of healthy ageing. Table 2.2 shows that older people had 9% higher body fat mass and 5% lower lean mass; mobility was reduced by at least 20%; handgrip strength was 22% lower and knee extensor strength was 35% lower than in young.

Muscle mass, bone density and physical activity levels
Sarcopenia leaves older people with limitations to their daily lives and at increased risk of falling. In older age, there is increased risk that a fall will result in a broken bone, due to the lower bone mineral density, leading to osteoporosis. In the MYOAGE cohort, the older men who maintained higher muscle mass and
strength also had higher bone density, but this was not the case in older women (Figure 2.1).

Figure 2.1. The association between different diagnostic criteria of sarcopenia and whole body bone mineral density (BMD) in young men (a), old men (b), young women (c) and old women (d). ALM in percentage is the appendicular lean mass as percentage of body mass. ALM percentage, ALM/height2 (kg/m2), knee extension torque (Nm) and walking speed (m/s) are presented in country, sex, and age group specific tertiles. Bars represent the adjusted means and s.e. P values were calculated with linear regression models with adjustments for age and country. *=p<0.01. **=p<0.001.

A possible explanation is that forces exerted by the musculo-tendon unit onto bone during physical activity serves as a stimulus for bone formation, so preservation of muscle size and strength as well as maintenance of physical activity levels has benefits also for bone tissue. The different results of older women could be due to different patterns of daily muscle activity or might be due to hormonal changes. In this type of research it is important to assess accurately habitual activity and there is a need to develop a more sophisticated, objective method to do so. Selections of accelerometry-based systems are commercially available to monitor physical activities, but they cannot always distinguish motorised transport from walking, running or cycling, and sometimes mis-classify physiologically-important physical activities. To overcome these limitations, Kayser Italia, working within WP2, developed the Personal Activity Monitoring System (PAMS). The developed computer software integrates accelerometer and heart rate data to classify intensity of movement. The global positioning signal is used to screen-out movements where the speed of travel is relatively high compared with accelerations and heart rate, thus indicating motorised transport. A prototype system is available at Kayser Italia, Italy.

Sarcopenia in the MYOAGE cohort

There are several definitions and diagnosis criteria for sarcopenia commonly used in research. Some definitions use muscle mass as the only variable; others include muscle mass and strength; some include total body mass or fat mass; while others additionally include mobility. These different criteria do not consistently identify the same individuals as sarcopenic. The original definition of sarcopenia was the loss of muscle mass with ageing (Rosenberg 1989), and the diagnosis proposed by Baumgartner (1998) was people who were > 2 standard deviations lower than the mean of younger adults when measuring Appendicular Lean Mass / Height2 (measured using dual energy x-ray absorptiometry). One of the striking features of the data presented in Table 2.1 was that total lean mass was reduced by just 5% in older subjects in our MYOAGE cohort. Using the definition of Baumgartner (1998), only 5% of the Workpackage 2 MYOAGE cohort were sarcopenic. Previous reports indicate sarcopenia prevalence to be around 15% of men and 24% of women aged 65-70 yrs, rising to >50% in people aged over 80 yrs (Baumgartner et al 1998; Iannuzzi-Sucich et al 2002). The difference between our MYOAGE cohort and the previous reports relating to the prevalence of sarcopenia is almost certainly due to differences in recruitment strategy, with our strategy to specifically target healthy older people.

The functional deficit of our older participants (see Table 2.1) contrasts sharply with the apparent modest loss of muscle mass.

Table 2.1: Participant characteristics, stratified by age (n=504).
Variables are presented as mean (standard deviation), unless indicated otherwise. For strength and performance measurements the best effort has been used for analysis. Independent samples t-tests were used to assess differences between young and old. a Data available in n=416. b Data available in n=420. c High alcohol use defined as for males > 21 units/week and females > 14 units/week. d Data available in
n=411. e Total lean mass as percentage of total body mass. f ALM (appendicular lean mass) as percentage of total body mass. g The highest value from the duplicate measurements has been used for analysis. g The fastest time from the duplicate measurements has been used for analysis. MMSE: mini mental state examination. GDS: geriatric depression scale. TUG: Timed Up and Go test.

Table 2.2. Mobility in relation to muscle size and function

This raises the very important and often controversial question of whether or not the conventional measurement of sarcopenia is appropriate. To address this question we included more detailed examination of the thigh using magnetic resonance imaging (Figure 2.2). MRI analysis of 80 subjects in Manchester, 100 in Paris and 50 in Tartu revealed that the quadriceps muscles of older people were on average 30% smaller than in young. This is of similar magnitude to the loss of quadriceps muscle strength in older age (35%). It is standard practice to normalise muscle mass to a measure of body size to allow comparisons to be made between people of different stature. Most often, muscle mass is normalised to height^2 or total body mass.

Figure 2.2. Magnetic resonance images of the mid-thigh. A young man is shown in A (age 24 yrs, with quadriceps muscles highlighted), an older man is shown in B (aged 80 yrs). All muscles were visibly smaller in the thigh of the older man, and also typical of ageing is the increased adipose tissue. Modified from McPhee et al 2013., Biogerontology

The problem with these normalisations is that height can be reduced by as much as 1 cm/yr in older age due to curvature of the spine and compression of vertebrae, thereby cancelling out the loss of muscle mass and thus, underestimating the extent of sarcopenia. Total body mass can fluctuate independently of lean mass due to changes in fat mass, thereby shifting the focus of sarcopenia away from muscle tissue and more towards total body composition. To overcome these problems, we developed an approach to normalise the quadriceps muscle volume to the femur volume in young and older MYOAGE subjects. The femur volume did not differ between young and old, thereby validating the use as an internal reference against which changes to muscle size can be related. This method of defining loss of muscle mass with ageing revealed that the quadriceps muscles were more susceptible to age-related atrophy compared with the hamstrings, adductors and abductor muscles of the thigh. It also identified 74% of older men and 58% of older women to be sarcopenic, which contrasts strongly with the data showing only 5% of older people were classified as sarcopenic according to the traditional classification method (Figure 2.3).

Figure 2.3A and B

Muscle weakness and reduced specific force with ageing

Muscle strength is proportional to the number of sarcomeres in parallel, which is represented by the physiological cross sectional area (PCSA) of the muscle. The smaller muscle size, or PCSA, of older people can explain most of the muscle weakness in older age. However, it can be seen in Figure 2.4a that the older people followed a different trend-line in the relationship of PCSA against maximal strength (MVC), indicating that for any given muscle mass, they had lower strength. This indicates that the older people had lower specific force, which is the force generated per unit muscle mass. We investigated differences between young and older adults in a comprehensive set of physiological and biomechanical
determinants of external force output to see which were contributing to weakness in old age. Young and old had similar levels of voluntary activation during MVC and the patella tendon moment arm was similar. Differences between age groups were evident in the quadriceps muscle fascicle length and pennation angle. After accounting for all of these factors, the quadriceps specific force was 16.5% lower in old compared with young (Figure 2.4b).

Figure 2.4A and B

Type I and II muscle fibres could have different specific tension, so we determined fibre type composition and cross sectional area using ATPase staining. The young and old had similar cross sectional area of type I fibres, but the type II fibres were on average 22% smaller in old compared with young. Single muscle fibres were isolated from the muscle biopsy specimens. Figure 2.5 shows that old had lower fibre cross sectional area (csa) in type 1 and 2 fibres; lower specific tension (Po/csa); and lower velocity of shortening (Vo). Effects of ageing were greater in type 2 fibres compared with type 1.

Figure 2.5. Analysis of isolated muscle fibres. Older muscle had lower fibre cross sectional area (csa) in type 1 and 2 fibres, lower specific tension (Po/csa) and lower velocity of shortening (Vo). Myosin concentration did not differ between young and old.

Although there was a tendency for old to have lower myosin concentration in isolated fibres, it was not statistically significant. We also found that older muscle contained higher levels of connective tissue and thus, lower concentration of contractile proteins in whole muscle. Costamere proteins were also lower in muscle samples of old compared with young (Figure 2.6). Older people tended to have higher levels of focal adhesion kinase (FAK) and its inhibitor FRNK, but markedly lower Vinculin and Tenascin-C compared with young. These costameric proteins are involved in helping to transmit the forces generated from the contractile proteins within the fibre to the extracellular environment of collagenous connective tissue.

Figure 2.6. Costameric proteins in young and older muscles. Cross indicates significant difference between groups.

Most of the force is transmitted linearly along the length of the fibre, but forces are also transmitted laterally from sarcomeres along the length of the fibre to the extracellular matrix. Together, these results indicate that the lower in vivo specific force of older muscle is related to reduced specific tension of type 1 and type 2 fibres, lower concentration of contractile proteins as well as reduced costameric proteins.

Skeletal muscle fatigue

We investigated the fatigability of quadriceps muscles because fatigue is a common complaint in older people and can impact on ability or willingness to complete daily tasks as well as increasing the risk of falling. In one fatigue test, participants were asked to hold a sustained isometric voluntary contraction at 50% of their maximal strength until task failure. Older people held the contraction for longer than young on average, but there was very large variability between people (Figure 2.7).

Figure 2.7. Older people held a sustained isometric knee extension for longer than young. There was very
large variability between people

In this type of contraction, the intramuscular pressure increases enough to occlude blood flow into the muscle, so provides little opportunity for metabolic recovery during the task. Fatigue in this instance is largely due to the rate at which ATP is utilised. More detailed examination showed that the superior fatigue resistance of old compared with young was associated with their having smaller muscles and slower contractile properties and thus, slower rate of ATP turnover. However, when contracting the quadriceps muscles using a series of 60 brief, intermittent stimulated contractions, the older and young subjects showed similar extent of fatigue, but women fatigued less than men (Figure 2.8).

Figure 2.8. Older and young people showed similar fatigue after 60 brief intermittent contractions. Women fatigued less than men.

Again, larger muscles with faster contractile properties tended to fatigue more quickly than smaller muscles with slower contractile properties. These results suggest that older people use ATP at a slower rate than young, but this did not translate to any benefit during repeated brief, intermittent contractions. One reason could be that the older people may have had a reduced rate of ATP recovery during brief rest intervals, the effect being to cancel out the advantage of slower rate of ATP utilisation. In order to resist fatigue, the ATP recovery needs to occur through oxidative pathways in the mitochondria. Western blot analysis of muscle biopsy samples showed old had lower concentration of key mitochondrial proteins compared with young (Figure 2.9).

Figure 2.9. Older people had lower levels of mitochondrial proteins compared with young.

This is indicative of lower oxidative potential to recover ATP in older muscle. Another factor that might influence the intermittent contractions is that shortening of muscle fibres against series compliance might lead to reduced efficiency of the older, slower, more compliant muscles.

The contributions of sarcopenia and other factors to loss of mobility with ageing

Demonstrating significant differences between young and old in neuromuscular characteristics is an important first step in understanding the deterioration of body systems evident even with ‘healthy’ ageing. However, it is necessary to determine the level of causality between the observed structural and functional changes to the neuro-musculo-skeletal systems with the loss of mobility in older age.

Stair negotiation: The risk of falling for older people increases when negotiating stairs, and falls on stairs is one of the main categories of domestic accidents. We completed experiments using a VICON motion analysis and instrumented staircase. The elderly were found to spend a longer time with their body weight retained on the trailing leg, keeping their centre of mass over the upper step for as long as possible. This strategy requires older people to produce higher relative and absolute joint moments around the ankle of the trailing leg, but the impact of the landing on the leading leg is less in absolute terms for the elderly, but when related to their muscle strength, the impact is similar in young and old. This can lead to a pattern of ‘sideways’ descent in older, weaker people, where the centre of mass is always over the upper step until the leading leg makes contact with the lower step. To determine whether the stepping strategy adopted by
the elderly was a direct consequence of their muscle weakness we mimicked the loss of strength in the elderly by adding 20% body weight to young subjects. When carrying the extra weight, young subjects changed the pattern of movement so they delayed the shift of centre of gravity, spending a longer time on the upper step and in the process reducing the load on the leading leg, while increasing that on the trailing leg. These results indicate that the problems experienced by older people in descending stairs are primarily due to a decrease in strength, which will be exacerbated by any increase in body weight as a consequence of increased body fat with ageing.

Walking and rising from a chair: We examined the association between sarcopenia and mobility. Diagnostic criteria for sarcopenia included relative muscle mass (total or appendicular lean mass (ALM) as percentage of body mass), absolute muscle mass (ALM/height² and total lean mass), knee extension strength, and handgrip strength. Physical performance comprised walking speed and Timed Up and Go test (TUG). In old participants, relative muscle mass was associated with faster walking speed, faster TUG, and a higher physical fitness. Knee extension torque was associated with a faster walking speed and better performance in TUG. In young participants, there were no significant associations between diagnostic criteria for sarcopenia and physical performance. These results indicate that relative muscle mass, defined as lean mass percentage or ALM percentage, as well as knee extensor strength were associated with physical performance. Absolute muscle mass including ALM/height² was not associated with physical performance.

The associations described here were not strong, and one of the limitations of the analysis was that the measurements of muscle size (completed using DXA) might not truly show the extent of sarcopenia in the muscles mainly involved in walking, as discussed in detail earlier in this report. Therefore, in 49 young (28 men; 22.4 ± 3.1y) and 66 older adults (31 men; 72.3 ± 4.9y) we additionally used available data from MRI to estimate thigh muscle and bone size as well as functional measurements of muscle power, voluntary activation, specific force and fatigue resistance. All of these factors were considered in relation to the performance during a 6-minute walking task (6MW) and the TUG, as measures of mobility. The MRI-based muscle:bone ratio revealed consistently stronger relationships with the mobility assessments. However, to understand whether this was a causal relationship it is necessary to plot results for young and old groups separately. When doing this, we found that the lower mobility of the older subjects compared to the young could not be attributed to differences in muscle size (at least in this sub-group of participants). This indicates that reduced muscle size is not the main cause of mobility limitations in healthy old, at least in this sub-group of the MYOAGE cohort. Similarly, strong correlations were seen between muscle strength (MVC) and mobility when considering all subjects (R² = 0.422 for 6MWD; 0.353 for TUG) and likewise for power and performance (R² = 0.460 for 6MW; 0.433 for TUG). But, the only relationship that remained significant when analysing data for young and old, men and women separately, was that both the 6MW and TUG were significantly and strongly correlated with power normalised to body mass in older women. For the older men, there was also a significant relationship between power normalised to body mass and 6MW, but this did not apply to TUG. There were no significant relationships for the young men or women, but this is not surprising because the tests are not designed to test functional capacity of young, healthy people in whom performance in 6MW and TUG is probably limited by biomechanical factors. Overall, the results suggest that the loss or muscle strength and particularly power during healthy ageing is a cause of reduced performance in mobility tests (walking and TUG). The lower power of older muscle was mainly due to slower velocity of movement. This could be related to altered neural activation, such as the level of voluntary activation or motor unit firing rates, but is probably mainly due to slowing of the
contractile function. This fits with our observations of slower muscle phenotype made when using electrical stimulation, and lower specific tension, shortening velocity and power of isolated muscle fibres, as described above. Altered tendon properties would also contribute to reduced velocity of movement, and thus, power.

Influence of the lower-leg characteristics on mobility in older age: Most of the efforts within our work focussed on the quadriceps muscle group, because they are highly susceptible to weakness with ageing and important for generating the strength and power for every-day movements. However, the mobility tests we used were dependent also on the function of the lower leg neuromuscular system to perform plantar and dorsi flexions while ambulating. In a sub-group (n=52) of participants the plantar flexion strength, medial gastrocnemius and soleus muscle architecture and Achilles tendon stiffness were examined for association with mobility. We found that older participants had 17% lower Achilles tendon stiffness and 32% lower Young’s modulus than young, while tendon cross-sectional area was 16% larger in old (Figure 2.10).

Figure 2.10. Achilles tendon and young’s modulus were lower in old compared with young. Triceps surae muscle size was smaller and gastrocnemius medialis muscle fascicle length shorter in old compared to young. Maximal plantar flexion force was associated with tendon stiffness and Young’s modulus. The results suggest that regardless of age, Achilles tendon mechanical properties adapt to match the level of muscle performance. Old people may compensate for lower tendon material properties by increasing tendon cross-sectional area. Partial correlations revealed that both 6MW and TUG were strongly related to plantar flexion strength, Achilles tendon stiffness and soleus fascicle pennation angle (Figure 2.11).

Figure 2.11. Association between characteristics of the lower leg and mobility.

In addition the 6MW was associated with medial gastrocnemius fascicle and soleus fascicle length. These correlations were consistently stronger than those found for the characteristics of knee extensors. This was confirmed using linear stepwise regression analysis that revealed plantar flexor strength was more strongly associated with 6MW and TUG than the more commonly reported knee extension strength or leg extension power. It was also observed that Achilles tendon stiffness and triceps surae muscle architecture predicted mobility independently of plantar flexion strength. These results indicate the importance of plantar flexors for mobility in older people.

Conclusions
The results of this work provide a detailed insight into the neuromuscular changes that occur during “normal, healthy” ageing. We have linked neuromuscular deterioration to the reduced mobility of older people and provided a molecular basis for some of the changes. The scientific data are retained in a Database that is held by all Partners involved in this workpackage. The remaining blood samples, including plasma, serum and white blood cells are stored in a biobank for future follow-up investigations.

Work Package 3
Stem cells and tissue niche: two faces of the same medal
The capacity of adult tissues to regenerate in response to injury stimuli represents an important
homeostatic process that until recently was thought to be limited in mammals to tissues with high turnover such as blood and skin. However, it is now generally accepted that each tissue type, even those such as nerve, cardiac and skeletal muscle that are considered post-mitotic, contains a reserve of undifferentiated progenitor cells, loosely termed stem cells, that participate in tissue regeneration and repair. Regeneration represents a coordinate process in which these stem cell populations are activated to maintain and preserve tissue structure and function upon injured stimuli. Nevertheless, the complete regenerative program in case of aging, extended injury or pathological conditions is severely affected and it precluded by fibrotic tissue formation and consequent functional impairment. In particular, increasing muscle weakness is a major component of muscle ageing. In the elderly, muscles become atrophic (loss in muscle mass) and weaker (loss in muscle force), more susceptible to damage and consequently regenerate and recover more slowly than was the case in their youth.

The Myoage project, related to WP3, aimed at understanding the modifications appearing with ageing in the behavior of the precursors, and may thus participate to muscle weakness by limiting nuclear turn-over, but mainly by slowing down muscle repair after trauma. The close collaboration of different partners involved in this effort, has defined the potential cellular and molecular mechanisms that can perturb progenitor function during ageing, and which ones can be the targets of action against muscle weakness. The regenerative capacity of the skeletal muscle is guaranteed by an intrinsic mechanism that restores the injured contractile apparatus. The dominant role in muscle homeostasis and regeneration is played by satellite cells. Historically, satellite cells were identified in 1961 using electron microscopy studies by Mauro, who reported that satellite cells might be “dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fibers when the main multinucleate cell is damaged”. Satellite cells are activated in response to both physiological stimuli, such as exercise, and under pathological conditions, such as injury and degenerative diseases, to generate a committed population of myoblasts that are capable of fusion and differentiation. Satellite cells are able to fuse to each other to form new myofibers or alternatively fuse with existing myofibers, repairing damaged muscle fibers (Figure 3.1).

Nevertheless, even though skeletal muscle possesses a stem cell compartment, which decreases with age in rat, mice and humans, it is not sufficient to explain why the aged muscle presents such a diminished efficiency to regenerate. Either the potency of the resident muscle stem cells drastically decreases during aging or perhaps the senescent muscle is a prohibitive environment for stem cell activation and function.

Figure 3.1 Model of satellite cells-mediated muscle regeneration

Senescent satellite cells display a delayed response to activating stimuli and show a reduced proliferative response to their sub-optimal environment.

Telomere shortening during proliferation may contribute to the inability of satellite cells to perpetually repair muscle during aging. Human satellite cells, like most somatic cells of the body, lack telomerase expression after birth, and their telomeres will shorten at each cell division until they reach a critical length which triggers proliferative arrest or senescence.

To understand if proliferative aging could induce modifications in the intrinsic properties of human myoblasts, as approach to senescence does in vitro, we have monitored the proliferative capacity of myoblasts isolated from young and old donors. As shown in figure 3.2A and as we reported in a recent publication in Biogerontology, satellite cells isolated from old adults were able to proliferate and to express specific markers of activated and proliferating satellite cells, such as desmin, Pax7, Ki67, and MyoD.
We also compared the proliferative capacity of satellite cells from 5 young donors (age range 15-24 year old) to that of 10 old donors (age range 68-80 year old) separated in 2 groups: 5 old sedentary and 5 old active based on their examination by clinicians and on a questionnaire on the lifestyle of the subjects. In conclusion of our experiments, cultures derived from old active or sedentary subjects did not show any significant difference in their proliferative capacity, nor do they differ from those derived from young donors. However, when cells were cultured in presence of 15% autologous or heterologous sera – which may represent more physiological conditions - we observed a significant difference in the rate of proliferation when old-derived satellite cells were compared with young-derived satellite cells (Figure 3.2B). In particular, the proliferative capacity of the muscle cell cultures was estimated from the cells incorporating BrdU. For this experiment, young- and old-derived satellite cells were plated at the same density and immunofluorescence analyses revealed that the percentage of BrdUpositive cells was reduced in cultures of old-derived satellite cells when cultured in autologous (homochronic) as compared to heterologous (heterochronic from young donors), or to young satellite cells (Figure 3.2B). The impaired satellite cells behaviour in sarcopenia might be mediated by altered p53 expression/activity. The tumor suppressor p53 is activated by different stress signals, such as DNA damage, leading to cell cycle arrest, apoptosis but also telomere shortening driven senescence. In agreement to these findings, we observed an increase in the expression levels of p53 and of its downstream gene p21, in myoblasts derived from old (age: 73.37 ± 2.66 year old) subjects with respect to young (age: 21.6 ± 2.23 year old) (Figure 3.2C). These data support the evidence that during aging, satellite cells display a delayed response to activating stimuli and show a reduced proliferative response to their environment when this environment is sub-optimal. Of interest was the observation that human satellite cells fail to differentiate when cultured in isochronic conditions. Immunofluorescence analysis for the expression of MyHC revealed that autologous serum (isochronic culture conditions) dramatically reduced muscle differentiation of aged satellite cells (Figure 3.2D) which was partially rescued when aged satellite cells were differentiated in heterologous/heterochronic serum (from young donors) (Figure 3.2D). This suggests that impaired efficiency of activation, proliferation and differentiation of satellite cells might be the result of the aging environment and not necessarily due to inherent changes in the cells themselves.

Figure 3.2 Aged satellite cells although are able to proliferate (A), display a delayed response to activating stimuli and a reduced proliferative response to their sub-optimal environment (B), which might be caused by the altered expression/activity of p53 pathway (C) and display a defect in muscle differentiation when cultured in autologous serum (D).

Heterochronic experiments have also demonstrated that old muscle successfully regenerates when transplanted in a young animal, whereas the regeneration of young muscle transplanted in an old host is impaired. This hypothesis has been clearly validated by parabiotic experiments, the union of two organisms that share the circulatory system, demonstrating the rejuvenation of aged progenitor cells by exposure to a young systemic. These results emphasize the importance of the environment, which is created by circulating factors, but also by the local secretome of factors secreted by the cells, such as satellite cells, the newly differentiating fibers, as well as by the inflammatory context of the early steps of muscle regeneration. In fact, epidemiological studies indicate that age-related decline of muscle mass and strength (sarcopenia) is associated with increased plasma level of proinflammatory markers (such as TNFalpha, IL-6, CRP), coupled to decreased levels of growth factors (IGF-1).
We also monitored the effects of different isoforms of IGF-1 on muscle regeneration. Our study assigned distinct functions to IGF-1 isoforms in skeletal muscle, and highlighted the role of E-peptides in tissue homeostasis and regeneration. We demonstrated that the major differences among the different isoforms of IGF-1 on muscle regeneration is related to the rapidity to rescue the injured phenotype. It is therefore clear and plausible that, accelerating the recovery of injured muscle, IGF-1 exerts a better anabolic effect, avoiding the generation of hostile microenvironment that might delay/compromise the functional rescue of injured skeletal muscle. All of these findings demonstrate that sarcopenia has a multi-factorial etiology, involving a great variety of structural, biochemical and physiological abnormalities. Moreover, with age, the systemic environment is less effective in maintaining the myogenic fate of muscle stem cells and, instead, facilitates conversion to a fibrogenic fate, while the myogenic stem cells may be more sensitive to their environment, thus amplifying this phenomenon. Thus, aging creates an hostile microenvironment that might impinge the physiological activity of resident and recruited stem cells (Figure 3.3). The modifications with aging in the secretome of the different cell types present at different phases of regeneration is probably then crucial, as is expression of relevant cell surface receptors, which can also change with age (e.g. IGF and IGF receptors). Thus, while stem cells represent an important determinant for tissue regeneration, a “qualified” environment is necessary to guarantee and achieve functional results. In this context, therapeutic applications of adult stem cells to aged or pathological tissue repair in the context of regenerative medicine will require an increased understanding of stem-cell biology, of the cross-talk between the different cell types involved and of the environment of the ageing or pathological tissue.

Figure 3.3. Schematic representation of pathologic alterations associated with aging, which create an hostile microenvironment that in turn affects the activity of stem cells.

The partners/research groups involved in this part of the Myoage project include:
• Gillian Butler-Browne’s group (Institut de Myologie, Paris, France)
• Musarò’s group (Sapienza University of Rome, Rome, Italy)
• Marco Narici’s group (University of Nottingham, UK)
• Claudio Franceschi’s group (University of Bologna, Bologna, Italy)

Work Package 4 (Signalling pathways)
The objective of WP4 was to characterize the signalling pathways responsible for the regulation of muscle mass during adulthood and ageing, with a view to identifying potential therapeutic target for the treatment of sarcopenia. The studies involved the following partners:
• Stefano Schiaffino and Marco Sandri from the Venetian Institute of Molecular Medicine, Padova (VIMM, P2),
• Mario Pende from the Institut National de la Santé et de la Recherche Médicale, Paris (INSERM, P1b),
• Antonio Musarò from the University of Rome (UNIROMA, P3),
• Pura Munoz-Canovés from the University Pompeu Fabra of Barcelona (UPF, P10),
• Rosario Rizzuto and Carlo Reggiani from the University of Padova (UNIPD, P11).
To determine the changes taking place in signalling pathways during ageing, we took advantage of the availability of muscle samples collected by other members of the MYOAGE consortium from well-characterized cohorts of young, old sedentary and old active individuals, and of muscles from mice aged 200, 500 and 800 days. To determine the effect of genetic perturbations of specific signalling pathways on muscle mass, we used in vivo transfection approaches and generated a number of transgenic and
knockout lines: we analysed the effect of these perturbations on muscle morphology (changes in fibre size), biochemistry (changes in mRNAs and proteins, activation/inactivation of specific pathways) and physiology (contractile performance in vivo). A global view of this collaborative project, involving all members of WP4, has been published in a recent review article (Sandri et al, Biogerontology 2013). Our studies investigated a variety of extracellular signals, including the plasminogen activator inhibitor 1 (PAI-1) and interleukin 6 (IL-6), as well as intracellular systems, including mitochondrial function. However, we will focus here on the myostatin and IGF1-Akt pathway, which are more directly relevant to muscle atrophy. Indeed, when the MYOAGE project started in 2009, the current view was that muscle mass is controlled by two major signalling pathways, the myostatin-Smad and the IGF1-Akt-mTOR-FoxO pathway (Fig. 4.1).

Figure 4.1. Scheme of the two major pathways involved in muscle mass regulation, based on the knowledge available when the MYOAGE project started in 2009.

Our first objectives were therefore to determine i) whether the components of these pathways are changed during ageing, thus altering the balance between protein synthesis and degradation leading to sarcopenia, and ii) whether it is possible to delay or prevent sarcopenia by interfering with the protein degradation pathways which are involved in muscle atrophy. We found that whereas myostatin levels are not significantly changed during ageing, there are some significant differences in the IGF1-Akt-mTOR-S6K pathway in ageing mouse skeletal muscle. In particular, the transcripts of IGF1 isoforms containing variable C-terminal E peptides, derived from alternative splicing of the Igf1 gene, are significantly reduced in skeletal muscles from very old mice (Fig. 4.2A). Another age-dependent change was the increased phosphorylation of S6, a ribosomal protein downstream of the Akt-mTOR-S6K pathway (Fig. 4.2B). The latter change is not easily explained, but may provide the rationale why rapamycin treatment and caloric restriction are beneficial in longevity, as these protocols blunt the activation of mTORC1. However, no significant age-dependent change in these two variables was detected in human muscle samples, presumably due to inter-individual variability.

Figure 4.2. IGF1 isoform distribution and S6 phosphorylation differ in skeletal muscle from very old (800-day-old) mice compared to adult (200-day-old) animals. (A) Skeletal muscles from very old mice show a difference in IGF-1 isoform expression, with significant reduction in Ea and Eb C-terminal E peptides. (B) Increased S6 phosphorylation in muscles from very old mice points to increased mTOR-S6K activity during ageing. (Sandri et al, Biogerontology 2013).

Next, we asked whether it is possible to spare muscle mass and reduce age-related muscle weakness by inhibiting the FoxO-dependent protein degradation pathways, the proteasomal and the autophagic-lysosomal systems. We previously reported that FoxO3A regulates the expression of the muscle-specific atrophy-related ubiquitin ligases atrogin1 and MuRF1 (Sandri et al, Cell 2004), therefore we examined muscles of atrogin1 and MuRF1 knockout mice during ageing. A significant sparing of muscle mass was observed in very old animals from MuRF1 knockout mice, whereas deletion of atrogin1 significantly reduced muscle weight. However, the lack of both atrophy-related ubiquitin ligases led to a decrease in muscle force.

Life span extension induced by caloric restriction requires an intact autophagy/lysosome system, a pathway important for the removal of damaged protein and dysfunctional organelles. To explore the role of autophagy in ageing skeletal muscles, we have generated a muscle-specific knockout model of the
autophagy gene Atg7 (Masiero et al, Cell Metab 2009) and found that both muscle mass and force are decreased in autophagy-deficient mice (Fig. 4.3). We also found that the lack of another autophagy-associated gene, Vps15, in skeletal muscles leads to autophagic vacuolar myopathy and lysosomal disease (Nemazanyy et al, EMBO Mol Med. 2013). In conclusion, these results strongly support the concept that the major proteolytic pathways, including the proteasomal and autophagic-lysosomal systems, are critical for the quality control of muscle proteins and need to be maintained active during ageing. Indeed, their chronic inhibition does not prevent muscle loss but instead exacerbates muscle weakness and should not be considered as a therapeutic target to counteract sarcopenia.

Figure 4.3. Muscle-specific knockout of the autophagy gene, Atg7, causes muscle atrophy (A), reduced muscle force (B), accumulation of protein aggregates visualized by p62 immunofluorescence (C), and accumulation of abnormal mitochondria with aberrant membranous structures visualized by electron microscopy (D). These findings indicate that the autophagy flux is important to preserve muscle mass and maintain myofibre integrity, whereas the inhibition of autophagy leads to myofibre degeneration and weakness (Masiero et al, Cell Metab 2009).

Finally, another major result of WP4 has been the recent discovery of the role of Bone Morphogenetic Proteins (BMPs) on muscle growth. This result emerged unexpectedly from studies on myostatin signalling. Myostatin is known to signal via transcription factors of the Smad family, specifically Smad2 or 3, which, upon phosphorylation by the activin receptor complex, dimerize with Smad4 and translocate to the nucleus acting on target genes (Fig. 4.4).

Figure 4.4. The scheme illustrates a novel concept in muscle growth regulation, which emerged during the last phase of activity of WP4. We found that muscle mass is controlled by the balance between myostatin signalling, acting as a negative regulator of muscle growth, and BMP signalling, acting as a positive regulator of muscle growth. Both myostatin and BMPs are TGF-β ligands and bind to type I receptors, such as activin receptor 2 (ACVR2) or bone morphogenetic protein receptor 2 (BMPR2), which in turn recruit type II receptors, such as ALK4 or ALK3. Active receptor complexes formed upon myostatin or BMP binding induce the phosphorylation of Smad 2/3 or Smad1/5/8, respectively. Phosphorylated Smad2/3 or Smad1/5/8 bind in turn to Smad4 and the heterodimers translocate to the nucleus and activate target genes, which differ between the two pathways and lead to opposite effects on muscle mass: activation of the myostatin pathway leads to muscle atrophy, while the activation of the BMP pathway leads to muscle hypertrophy. The analysis of these pathways is complicated by the existence of multiple ligands (myostatin-like factor, such as activin A, and multiple BMPs) and antagonistic factors (myostatin antagonists, such as follistatin, and BMP antagonists, such as noggin). (Sartori et al, Nature Genet, in press).

Previous studies showed that shRNAs against Smad2 and 3 promote muscle growth and prevent denervation atrophy. Sandri and collaborators have now generated a muscle-specific knockout of the Smad4 gene, and found that, surprisingly, mice with Smad4 mutations display muscle atrophy, rather than the expected muscle hypertrophy (Sartori et al, Nature Genet 2013). A series of investigations aimed to explain this puzzling effect led to the discovery that a signalling pathway mediated by another class of circulating factors, BMPs, acting through Smad1/5/8, which also dimerize with Smad4, is the fundamental hypertrophic signal. Inhibition of BMP signalling, using the BMP antagonist Noggin, causes muscle atrophy, abolishes the hypertrophic phenotype of myostatin knockout and strongly exacerbates the effects
of denervation and fasting (Fig. 4.5). In contrast, transfection with a constitutively active ALK3 construct causes muscle hypertrophy. The BMP-Smad1/5/8 pathway negatively regulates the Fbxo30 gene, encoding a novel ubiquitin ligase, Muscle Ubiquitin-ligase of SCF complex in Atrophy-1 (MUSA1), which is required for muscle loss. The identification of the critical role of the BMP pathway opens a completely novel perspective in the study of adult muscle maintenance, growth and atrophy, and may lead to new approaches for the treatment of sarcopenia.

Figure 4.5. A. Inhibition of the BMP pathway leads to muscle atrophy. Muscles transfected with Noggin (BMP antagonist) show decreased myofibre size. B. Activation of the BMP pathway induces muscle hypertrophy. Intra-muscular overexpression of a constitutively active type I BMP receptor (c.a.ALK3) leads to muscle hypertrophy in adult mouse muscles and prevents muscle atrophy after nerve section. (Sartori et al, Nature Genet, in press)

We would like to conclude this brief survey by emphasizing an additional, major role of WP4 activity, namely the formation of personnel. A large number of PhD students and postdocs have been introduced to the techniques of signalling biology, muscle biology and ageing science. It has been a pleasure to see that during this period some postdocs have become independent PIs. For example, Bert Blaauw, previously postdoc in Schiaffino’s lab at VIMM in Padova, has recently obtained a position of Assistant Professor in the Department of Biomedical Sciences of the University of Padova, and has now his own lab as Junior PI at VIMM.

Work Package 5

WP5 is centred on the assessment of the status of chronic, sub-clinical inflammation occurring during ageing, which we propose to define as “inflamm-aging”, and its consequences for the development of sarcopenia and muscle weakness with age. Many other studies directly or indirectly correlated with muscle inflammation have been performed in the framework of this WP, that has produced a great number of results related to the 12 Tasks in which it was subdivided, as summarised below.

A comprehensive analysis of the inflammatory status was performed by analysing a number of pro- and anti-inflammatory cytokines, adipokines and hormones, including IL-10, IL-6, TNF-alpha and soluble IL-1Ra (P4.1) adiponectin, leptin, resistin, ghrelin, as well as insulin and IGF-1 (P6). The data have been provided to the centralized database (P05) in order to be stratified with all the other parameters collected, including the activity status (active or sedentary), the health status, the muscle strength, etc. The analysis of other cytokines and neuropeptides is ongoing. The analysis of the plasmatic levels of adipokines in 412 subjects of different age (152 subjects aged 18-30 years and 260 subjects aged 69-81 years) has revealed that the levels of adiponectin (both in male and female subjects) and leptin (only in males) were significantly higher in old subjects compared to young, while those of IGF-1 were lower in old subjects. Moreover, for the first time a correlation was found between the levels of adiponectin, resistin and the resistin/IGF-1 ratio (but not IGF-1 alone) with muscle strength in old subjects. In particular they were inversely associated with quadriceps torque, while adiponectin was also inversely associated with handgrip strength. These associations were independent from percentage of fat mass, height, age, gender and geographical origin (Bucci et al., 2013). It is not yet clear the mechanism by which the circulating levels of these molecules could affect muscle strength, but all of them impinge upon fatty acid metabolism and storage, and it is known that intermuscular adipose tissue (IMAT) characterises sarcopenia and is related to muscle weakness. As expected IGF-1 levels were lower in old subjects with respect to young
P17 has evaluated several inflammatory factors in the subjects recruited within WP9 i.e. postmenopausal monozygotic female twin pairs discordant for estrogen based hormone replacement therapy (HRT) and premenopausal controls. The serum levels of adiponectin, monocyte chemotactic protein-1, and leptin, as well as their local transcript levels in adipose tissue, skeletal muscle, and leukocytes, were measured. As a whole, long-term HRT is associated with healthier amount and distribution of body fat and a better adipocytokine profile i.e. a 15% lower serum level of monocyte chemotactic protein-1 in HRT users (Ahtiainen et al., 2012). Regarding IL-6 it was shown that the systemic levels of IL-6 receptors sIL-6R and sgp130 were 16% and 52% (p≤0.001 for both variables) higher in postmenopausal women than in premenopausal women, and 10% and 9% lower (p = 0.033 and p< 0.001 respectively) in the HRT users than in their non user co-twins (Ahtiainen et al., 2012). After adjustment for body fat amount, the differences were no more significant. Secondly, the transcript analyses emphasize the impact of adipose tissue on systemic levels of IL-6, sgp130 and sIL6R, both at pre- and postmenopausal age. Furthermore, a strong negative association (r= -0.71) was found between serum IL6-R and IGF-1 in HRT using postmenopausal women, but not in their non-using identical co-twins. It can be summarized that in postmenopausal women the inflammatory status may be associated with the IGF-1 resistance in skeletal muscle. HRT potentially resists the enhancement of inflammation by increasing the amount of IGF-1R and by stimulating the post-receptor signalling.

A study on the activation of NF-kB in subjects of different age and level of physical activity has been performed in order to detect the status of inflammation at the level of the muscle cells. Indeed, also in muscle NF-kappaB plays an important role for inflammation and its activation appears to be significantly elevated by age (Buford et al., 2010). P6 investigated the p65 subunit of NF-kappaB complex and its phosphorylated active form in the biopsies of v. lateralis of healthy subjects and patients with lower limb mobility impairment (LLMI). Our results indicate a slight increase of p65 phosphorylated form in patients with respect to healthy subjects without any apparent age effect (Barberi et al., 2013).

A sensor of oxidative stress is p53, best known for its role of “guardian of the genome”. P53 can indeed be activated in response to a series of stress signals and damages, including DNA strand breaks and ROS. It plays a role in the maintenance of muscle quality but also in muscle wasting, owing to its pleiotropic effects in networks involved in energy production, including muscle mitochondrial biogenesis, fundamental for muscle performance, as observed in both mice and men. On the other hand, p53 is a well-known regulator of apoptosis, and this function is present also in muscle cells. We observed a substantial difference in the amount of active p53 between samples from healthy subjects and patients with LLMI, with the higher amount of active p53 being detected in old patients (Conte et al., 2013).

In the framework of the analyses on NF-kB and p53 activation, a study on the role of lipid droplets deposition within muscle fibres during aging and physical inactivity have been also performed. In particular, P6 performed a study on muscle biopsy from both healthy subjects and patients with LLMI regarding the expression of perilipin 2 (Plin2). Plin2 is a muscle-specific member of the perilipin family of proteins that are associated with intracellular lipid droplets. While the majority of the available data emphasize the role of Plin2 for the mobilisation of fatty acids, nothing is known regarding the possible modulation of Plin2 expression with age, and its correlation with muscle strength in old people. In contrast with the current knowledge on the positive role of Plin2 in exercised people, we have found a dramatic increase in Plin2 expression in v. lateralis of old people, and especially in old patients with LLMI, with respect to younger counterparts, that was correlated with p53 activation. Strikingly, Plin2 expression was inversely correlated with quadriceps thickness and strength, and this effect was counteracted by IGF-1 in
animal models. These data suggest that aging and, in part, physical inactivity play a role in determining Plin2 expression (and therefore intramuscular fat deposition), and that this contributes to muscular weakness (Conte et al., 2013).

Figure 5.1. Representative example of the correlation between muscle thickness, measured by ultrasound analysis, and Plin2 expression (brown dots of Immuno histochemistry, IHC, images) in v. lateralis from young and old subjects.

The possible role of inflammation in muscle repair and regeneration or fibrosis as well as in myoblast proliferation and differentiation has been studied. In one study, the ablation of p38α in inflammatory cells has been investigated in order to understand the role of this protein in the muscle regeneration-promoting activity of these cells. To this purpose, P10 generated conditional knockout mice deficient in p38α in the inflammatory compartment by intercrossing p38α(f/f) mice with Lys-CRE mice. The specific deletion of p38α in the inflammatory compartment did not alter significantly fiber type distribution or myofiber size in basal conditions suggesting that p38α expression in the inflammatory compartment is dispensable for muscle formation and developmental muscle growth.

To characterize whether ablation of p38α in this compartment affects muscle regeneration, adult p38αWT and p38αΔLys were subjected to muscle injury and the regeneration process was characterized. Five days after CTX injection, the process of muscle regeneration was affected significantly by the lack of p38α in the inflammatory compartment as demonstrated by mean fiber size and fiber size frequency distribution. Future experiments will determine if this result will be correlated in aged mice.

To further characterize the role of p38α in the inflammatory compartment in satellite cell activation hindlimb muscles of p38WT and p38ΔLys mice 18 hours post-injury were processed for cell sorting analysis and satellite cells were obtained. The myogenic capacity of these cells was analyze by Immunostaining and no significant difference was observed for Pax7/MyoD double positive cell number. Future experiments will determine if this result will be correlated in aged mice.

MiRNAs are fine-tuning regulators of skeletal muscle development, function and aging, but knowledge of their hormonal control is lacking. P17 and P6 used a co-twin case-control study design, i.e. monozygotic post-menopausal twin pairs discordant for estrogen-based Hormone Replacement Therapy (HRT), to explore estrogen dependent muscle regulation via miRNAs. MiRNA profiles were determined from v. lateralis muscle of nine healthy 54-62-year-old female twin pairs discordant for HRT (median 7 years). Among miRNAs expressed in muscle samples miR-182, -223 and -142-3p showed significantly lower expression levels in HRT users compared with their non-user co-twins. “IGF-1/Insulin signalling” emerges as common pathway targeted by these miRNAs. Accordingly, we found that members of this pathway such as IGF-1R and FOXO3A are more abundantly expressed in the muscle samples of HRT users than in non-users. In vitro assay confirmed the effective targeting of miR-223 and -182 on IGF-1R and FOXO3A mRNAs. Moreover, Estradiol treatment of MCF-7 cell line, confirmed a dose-dependent miR-182 and -223 down-regulation, as well as FOXO3A and IGF-1R mRNA and protein level up-regulation. We conclude that HRT has positive effects on skeletal muscle of postmenopausal women likely by up-regulation of the IGF-1 pathway mediated by the reduction in the expression of miRs-182, -223 and -142-3p (Olivieri et al., submitted).

P16 characterized the effects of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) on differentiation and
energy metabolism of myoblasts. The cultures of myoblasts obtained from m. vastus lateralis biopsies of young and old individuals were stimulated for 6 days with each cytokine (10 ng/ml) alone or in combination with differentiation media supplement, insulin- transferrin-sodium selenite. Differentiation was estimated by monitoring myotube formation, fusion index and % of myonuclei. The differentiation efficiency was also assessed by the specific markers for muscle cells; the expression level of muscle-type creatine kinase and myogenin was significantly higher in the myotubes compared to myoblasts. The myoblasts cultivated from old individuals differentiated into myotubes markedly slower than myoblasts from young individuals in ITS medium (p<0.0001). IL-6 alone induced the differentiation to the same level as ITS and there was no remarkable co-effect of IL-6 with ITS detected. The effect of IL-6 depended on donor age, as its effect on myoblast differentiation decreased with age. Treatment of human myoblasts with TNF-α and IL-1β increased the proliferation and blocked differentiation even in the presence of differentiation media supplement (ITS). The inhibitory effect of TNF-a and IL-1β on myotube formation was mediated by down-regulation mRNA level of myogenin and muscle-specific isoforms of CK (CKM and CKMT2). Oxygraphic measurements of mitochondrial respiration were performed by P16 on myoblast cultures obtained from biopsies of young (n=15) and old (n=19) subjects. IL-1β caused a significant decrease in complex I and II respiration normalized on protein content both in the myotubes of old and young individuals (Fig. 5.2). This action of IL1-β was not seen if the results of respiration were normalized on citrate synthase activity, revealing a decrease in the mitochondrial content in these cells (Fig. 5.3). TNF-α, on the contrary, caused a significant increase in complex I and II respiration normalized on protein content in myotubes of old and young individuals. This action of TNF-α was significant also in case if the results of respiration were normalized on citrate synthase activity. Thus, the activation in OXPHOS was stimulated by the increase in the biogenesis of mitochondria and the stimulatory effect on respiratory chain. IL-6 exerted no alteration on the respiration of the myotubes. Comparison of the effects of TNFα, IL-1β and IL-6 on functional coupling between OXPHOS and hexokinase (HK) in myogenic cells show that the control myotubes forming under the influence of ITS exhibited a marked stimulation of the state 3 respiration by glucose, this indicating an interaction between HK and mitochondria. IL-1β and TNF-α increased more the effect of glucose in comparison of ITS treated cells. Our results suggest that suppression of the differentiation of myotubes by TNF-α or IL-1β was associated with increased functional coupling between OXPHOS and hexokinase (HK) in these cells. Altogether these results suggest that suppression of differentiation of myotubes by TNF-α was associated with marked rearrangement of mitochondrial and glycolytic systems, in favor of forming network between them. P16 found that IL-6 tended rather to suppress than stimulate the coupling of HK to OXPHOS. In non-differentiated myoblasts the activity of creatine kinase (CK) was 3 times lower than in myotubes. Thus, differentiation of myoblasts to myotubes significantly increased activity of CK in these cells. Treatment of human myoblasts with TNF-α and IL-1β that increased the proliferation and blocked the differentiation in the presence of differentiation media supplements (ITS) inhibited also the rise of CK activity. IL-6 added to myoblasts that promoted differentiation, did not prevent the rise of CK activity during the differentiation. Responses of myogenic cells derived from m. vastus lateralis biopsies between young and old individuals did not differ in response to proinflammatory cytokines. The function of mitochondria in myogenic cells is strongly controlled by fluctuations in cytosolic Ca2+. P16 have shown that increasing [Ca2+] has dual effect on mitochondrial function: in lower range Ca2+ stimulates OXPHOS, but at higher concentrations suppresses it, most likely due to opening of the PTP channel. The maximum stimulating effect of Ca2+ on OXPHOS was about 30% in both groups, young and old myotubes. In both groups the IL-1 and TNF-α opposed the Ca2+ induced activation. There was a significant decrease in the Ca2+ induced activation among myogenic cells treated
with IL-1β or TNF-α.

Figure 5.2. Comparison of the effects of TNF-α, IL-1β and IL-6 on respiratory parameters of the myogenic cells of the old and young persons cultured either in the presence of ITS (control, +ITS) or ITS and one of three cytokines. The respiratory parameters were normalized on protein.

Figure 5.3. Comparison of the effects of TNF-α, IL-1β and IL-6 on respiratory parameters of the myogenic cells of the old and young persons cultured either in the presence of ITS (control, +ITS) or ITS and one of three cytokines. The respiratory parameters were normalized on the content of citrate synthase (CS). Inflammation and production of Reactive Oxygen Species (ROS) are strictly related, and ROS have a plethora of effects on muscle cells. A study performed within this WP by P11 discovered that ROS have a strong adipogenic transdifferentiating effect on muscle cells, and that p66shc is an important mediator of such an effect. In the same framework, as mitochondria are the main source of ROS, and also serve as Ca++ store, it has been studied how the flux of Ca++ ions is regulated, and this led to the discovery of the Mitochondrial Calcium Uniporter channel (MCU). The increase in ROS production stimulates a pleiotropic response in mammalian cells, ranging from protein and lipid modification, regulation of gene expression, alteration of metabolism and activation of apoptosis. In the previous two years, we showed that induction of ROS production is a potent stimulus for adipogenic differentiation in muscle tissue. High glucose treatment, which mimics the hyperglycemia condition in primary muscle stem cell culture, and H2O2 or glycerol injections in mouse muscle were the approaches we used to increase the level of ROS in vitro and in vivo, respectively. In both cases, we observed a significant correlation between ROS production and the trans-differentiation of muscle cells into adipocytes.

P11 focused on the analysis of the effects of High Fat Diet feeding (HFD: 60% of calories coming from fat) as an additional experimental model to stimulate ER stress and ROS production in muscle tissue in vivo. We previously reported that ER stress markers (SREBP and XBP1 proteins) are expressed at slightly higher level in p66shc−/− compared to wt muscle in basal condition. However, the expression of these proteins dramatically increases in wt animals after muscle injury (H2O2 and glycerol injection). This increase is blunted in p66shc−/− animals. We think that the higher level of basal ER stress signaling in p66shc−/− muscle in some way protects them from the eventual up-regulation of this pathway induced by muscle injury. The reduced activation of the ER stress pathway would then prevent ROS- and glycerol-induced fatty degeneration in p66shc−/− muscle. We wanted to demonstrate if this is the case also in the muscles of diet-induced obese mice. To accomplish that, we administrated HFD to wt and p66shc−/− mice for 4, 6 and 10 months and we analyzed body weight gain and the expression of adipogenic markers, ER stress and ROS signaling proteins in skeletal muscle at each time point. As already shown (Berniakovitch et al, JBC 2008), p66shc−/− mice showed a reduced weight gain in compared to wt mice. The increase in body weight in p66shc−/− was 30%, 44% and 50% less than wt at 4, 6 and 10 months respectively. P11 analyzed the transcript level of some adipocyte-specific markers (adiponectin, leptin and C/EBPa) by real time PCR and we found that only after 10 months, but not at 4 and 6 months of HFD feeding, there is a significant up-regulation of adiponectin and leptin expression in wt muscle compared to chow diet feeding. Interestingly, this up-regulation does not occur in p66shc−/− muscle, thus indicating that p66shc is required for the adipogenic differentiation of HFD treated muscle. P11 also looked at the expression of ER stress proteins (SREBP, XBP1 and CHOP) in HFD wt and p66shc−/− muscles. Also in HFD fed mice, as it was for chow fed animals, p66shc−/− muscles express higher level of ER stress proteins compared to wt in basal condition. However, the increase in expression
of these proteins after muscle injury (glycerol or H2O2 injection) observed in wt HFD muscle is significantly blunted in p66shc-/- HFD muscle. This indicates that p66shc modulates the activation of the ER stress response and that it is involved in the regulation of adipogenic differentiation in skeletal muscle. We now need to to determine the molecular mechanisms and the transcriptional factors that mediate the action of p66shc on the expression of ER stress- and adipocyte-specific genes.

Mitochondrial Ca2+ homeostasis is an important component of the calcium-mediated cellular response to extracellular stimuli. While the physiological and pathological relevance of mitochondrial Ca2+ homeostasis is fully appreciated, lack of molecular insight has severely limited mechanistic understanding and pharmacological targeting. P11 have recently identified the “mitochondrial Ca2+ uniporter” (MCU), the IMM channel that allows Ca2+ entry into the mitochondrial matrix (De Stefani et al. Nature, 2011, 476: 336-340). We have identified in silico a protein that shares tissue distribution with MICU1, a recently characterized uniporter regulator, is present in organisms in which mitochondrial Ca2+ uptake was demonstrated and whose sequence includes two transmembrane domains. Short interfering RNA (siRNA) silencing of MCU in HeLa cells markedly reduced mitochondrial Ca2+ uptake. MCU overexpression doubled the matrix Ca2+ concentration increase evoked by IP3-generating agonists, thus significantly buffering the cytosolic elevation. The purified MCU protein showed channel activity in planar lipid bilayers, with electrophysiological properties and inhibitor sensitivity of the uniporter. A mutant MCU, in which two negatively charged residues of the putative pore-forming region were replaced, had no channel activity and reduced agonist-dependent matrix Ca2+ concentration transients when overexpressed in HeLa cells. p66Shc translates oxidative damage into cell death by acting as reactive oxygen species (ROS) producer within mitochondria. Oxidative stress induces phosphorylation of p66Shc and triggers mitochondrial accumulation of the protein. Once imported, p66Shc causes alterations of mitochondrial Ca2+ responses and three-dimensional structure, thus inducing apoptosis. Thus p66shc is part of a signaling route that activates apoptosis and could be a potential target of pharmacological approaches to inhibit aging. We hypothesized that p66shc deletion may confer resistance to apoptotic stimuli in part by regulating MCU and/or MICU. Protein expression analysis revealed that MCU and MICU1 are less expressed in p66shc KO tibialis anterior muscles, suggesting that, at least in part, p66shc muscles may be protected from stress stimuli by suppressing two key components of mitochondrial Ca2+ entry machinery.

The mitochondria are the sole organelles that possess their own DNA (mtDNA), and it tends to accumulate somatic mutations and deletions. Age appears to affect both mitochondrial DNA (mtDNA) content and integrity. The decline in mtDNA copy number is also accompanied by an increase in mtDNA damage, particularly deletions and oxidative lesions, but also point mutations, tandem duplications, and rearrangements. P6 investigated mtDNA copy number and deletion level on muscle biopsy of v. lateralis from both healthy subjects and patients with LLMI, and observed that in patients the copy number decreased with age, while the deletion level did not change. Interestingly, a gender difference emerged: mtDNA copy number decreased with age in male patients, but not in females, while the deletion level increased with age in males, but decreased in females. These data have been correlated with muscle strength and we found that only in male patients high values of quadriceps torque correlate with high levels of mtDNA copy number. Conversely, no significant differences have been found in mtDNA copy number and deletion levels by age and gender in healthy subjects. Little is known on the amount and quality of mtDNA in myoblasts. P6 measured the copy number and the deletion level of mtDNA of 11 cultures of myoblasts obtained from young (3), old active (4), old sedentary (4) subjects cultured at 5% or 21 % O2 concentrations. No difference emerged for either copy number or deletion level, either when comparing different oxygen percentages within the same age-range, or when comparing different age groups within
the same oxygen percentage. However, we found that the deletion level significantly decreases and the copy number increases, but only in cells cultured at 5% O2, suggesting that independently from the age of the donor, the low oxygen percentage environment could favour the replication of complete mtDNA molecules.

During studies addressed to reveal novel molecules involved in the fibrinolitic/fibrin system in muscular dystrophy progression, P10 identified the extracellular PAI-1/urokinase-type plasminogen activator (uPA) balance as an important regulator of microribonucleic acid (miR)–21 biogenesis, controlling age-associated muscle fibrosis and dystrophy progression (Ardite et al, 2012). Genetic loss of PAI-1 in mdx dystrophic mice anticipated muscle fibrosis through these sequential mechanisms: the alteration of collagen metabolism by uPA-mediated proteolytic processing of transforming growth factor (TGF)-β in muscle fibroblasts and the activation of miR-21 expression, which inhibited phosphatase and tensin homologue (PTEN) and enhanced AKT signaling, thus endowing TGF-β with a remarkable cell proliferation–promoting potential. Age associated fibrogenesis and muscle deterioration in mdx mice, as well as exacerbated dystrophy in young PAI-1−/− mdx mice, could be reversed by miR-21 or uPA-selective interference, whereas forced miR-21 overexpression aggravated disease severity. The PAI-1–miR-21 fibrogenic axis also appeared dysregulated in muscle of DMD patients, providing a basis for effectively targeting fibrosis and muscular dystrophies in currently untreatable individuals.

To provide direct evidence for a regulatory role of miR-21 in skeletal muscle fibrosis, WT-lacerated muscles, and muscles of aged mice were subjected to a miR-21 modulatory treatment by miR-21 inhibition (antagomiR-21, hereafter referred to as Ant-miR-21) or miR-21 overexpression (Mimic-miR-21) for 1 or 4 wk, respectively. Ant-miR-21 treatment reduced miR-21 expression (but not the expression of an unrelated miR, miR-146) in the muscle of both mouse fibrotic models, whereas delivery of a scrambled oligomiR (Scramble) or a validated point mutant of Ant-miR-21, termed Ant-miR-21 U/C3, had no effect. Consistent with the blunted miR-21 expression, treatment with Ant-miR-21 (but not with a scrambled oligomiR) prevented the appearance of fibrosis-indicative parameters in lacerated WT muscle, and, more importantly, these fibrotic indicators were also reversed by Ant-miR-21 treatment in limb muscles of 24-mo-old mdx mice (at the stage at which mdx fibrosis is generally considered irreversible. Conversely, the overexpression of miR-21 by intramuscular administration of a miR-21 mimic anticipated and exacerbated fibrosis in lacerated muscles of WT mice and in young mdx mice (3 mo old). These results demonstrate the efficacy of miR-21 silencing in preventing and treating muscle fibrosis. Notably, miR-21 interference for 1 mo in very old mdx dystrophic mice also reduced muscle deterioration. As affected individuals with prominent fibrosis at advanced disease stages of DMD represent the vast majority of patients and no treatment for efficiently reducing muscle fibrosis is yet known, these results undoubtedly have a strong therapeutic potential. Together, these data suggest that a certain level of pericellular PAI-1 is needed to avoid rapid fibrosis progression in injured and dystrophic muscle. Complete loss of PAI-1 results in unrestricted activation of uPA/plasmin in damaged and dystrophic muscle, leading to the unscheduled accumulation of collagen and fibrosis. More importantly, our results show that fibrosis in aged mdx mice, despite being considered irreversible, can be attenuated by specific miR-21 (or uPA) genetic–interfering treatments, improving muscle homeostasis, with potential clinical implications for DMD patients at advanced fibrotic stages.

Our results to define the role of fibrin in the inflammatory status of aging muscle (Vidal et al, 2012), show that fibrin deposition is a conspicuous consequence of muscle-vascular damage in dystrophic muscles of
DMD patients and mdx mice and that elimination of fibrin(ogen) attenuated dystrophy progression in mdx mice. These benefits appear to be tied to: (i) a decrease in leukocyte integrin αMβ2-mediated proinflammatory programs, thereby attenuating counterproductive inflammation and muscle degeneration; and (ii) a release of satellite cells from persistent inhibitory signals, thereby promoting regeneration. Remarkably, Fibγ390-396A mice expressing a mutant form of fibrinogen with normal clotting function, but lacking the αMβ2 binding motif, ameliorated dystrophic pathology. Delivery of a fibrinogen/αMβ2 blocking peptide was similarly beneficial. Conversely, intramuscular fibrinogen delivery sufficed to induce inflammation and degeneration in fibrinogen-null mice. Thus, local fibrin(ogen) deposition drives dystrophic muscle inflammation and dysfunction, and disruption of fibrin(ogen)-αMβ2 interactions may provide a novel strategy for DMD treatment.

The effect of exercise on the inflammatory status of elderly subjects has been studied. P4.1 have previously reported that anti-inflammatory treatment vs. placebo did not influence the response of satellite cells to a period of resistance training in 60+ individuals (Petersen et al., 2011). A cross-sectional study of old trained master athletes (O-Tr), healthy age-matched untrained controls (O-Un), young trained (Y-Tr) and young untrained (Y-Un) men has been performed. Values (mean and SEM) for quadriceps cross sectional area (Q-CSA) and plasma levels of C-reactive protein (CRP) are shown in Fig. 5.4 (left panel), where it appears that the O-Tr have similar levels to the Y-Un, suggesting that life-long endurance running prevents both the increase in circulating levels of inflammatory markers and the reduction in muscle size seen in aged untrained individuals. Also, in untrained old individuals (O-Un) there was a significant difference between the satellite cell content of type I and II fibres (p<0.01) Fig. 5.4 (right panel). There was a trend towards a similar difference in O-Tr (p=0.054) suggesting that lifelong endurance training cannot prevent the onset of the age-associated difference in satellite cell distribution between type I and type II fibres. There was however a strong positive association between fibre area and satellite cell content in trained individuals, suggesting that endurance training regulates the satellite cell content of healthy skeletal muscle in young and old individuals.

Figure 5.4.
The potential of Angiotensin II inhibition to shift the balance in signaling from a negative fibrotic response to a favourable myogenic response during adaptation to exercise (as illustrated in the figure 5.5) has been studied. The experimental intervention has been completed. 26 healthy elderly (64 years +) were recruited and assigned to ingest placebo or losartan (Angiotensin II receptor blocker) for a period of 1 week and we collected a blood sample and a muscle biopsy (“control leg”) before and after this week of treatment. The subjects then performed a single bout of hard resistance exercise comprising concentric and eccentric loading of the quadriceps muscle of one leg. A blood sample and a muscle biopsy (from the exercised leg) were collected 2.5 hours after completion of the exercise and on days 1, 4, and 7 after exercise. Muscle biopsies are currently being analysed by immunohistochemistry for the satellite cell response.

Figure 5.5. Hypothesised effect of Angiotensin II blocker on muscle.

The partners/research groups involved in this part of the Myoage project include:
• Claudio Franceschi’s group (University of Bologna, Bologna, Italy, P6)
• Bente Pedersen’s group (Institute of Sport Medicine, Copenhagen, Denmark, P4.1)
• Michael Kjaer’s group (Righospitalet Copenhagen, Denmark, P4)
• Andrea Maier’s group (Leiden University Medical Centre, Leiden, The Netherlands, P8)
Among the modifications that have been observed during ageing both at the cellular and organ levels, the post-translational modifications of proteins have been particularly well described. At the cellular level, an age-related accumulation of modified proteins has been associated with an impairment of proteasome function with aging. Skeletal muscle is composed of many proteins specialized for the contraction but also enzymes whose correct activity is required for the proper regulation of contraction. Knowledge on this aspect is sparse regarding ageing skeletal muscle. These mechanisms have been investigated in WP6, in order to get a complete and general view of the modification and turnover of proteins and their involvement in muscle weakness.

Proteomic analyses have been performed to determine biomarkers revealing changes either in the whole muscle fragment or at the cellular level looking both at protein level and post-translational modifications such as oxidation (carbonylation, glycoxidation) and phosphorylation. This has been addressed as a function of age and/or upon intervention aimed at counteracting ageing induced sarcopenia such as electro-stimulation training. Post-translational modifications of specific skeletal muscle proteins such as myosin have been also monitored as a function of age in animal and cellular models as well as in human biopsies.

Finally, both the lysosomal and proteasomal pathways, which represent the two main systems of intracellular protein degradation, have been investigated for identification of specific substrates in aging sarcopenia using animal models and in muscle cell cultures while age-related changes of proteasome function have been addressed in whole human muscle biopsies and muscle progenitors cells.

Identification of potential biomarkers of muscle aging based on whole proteome analyses approach

Top-down differential proteomic approaches have been undertaken to identify novel biomarkers for skeletal muscle aging. Muscle samples were compared between mature (60 + 1 years old) and old (78 + 2 years old) post-menopausal women. Both total muscle and sarcoplasmic extracts were investigated, and different methodologies of two-dimensional gel electrophoresis (2DGE) and shotgun proteomics were developed. Using these quantitative 2DGE proteomic approaches, out of a total of 1220 spots, 67 were found differentially expressed in old compared with mature muscles. Mass spectrometry (nanoLC-MS/MS) finally identified 48 spots, which corresponded to 39 different proteins in whole muscle extracts.

To improve the detection of low abundance proteins, muscle sarcoplasmic proteins were further investigated using both 2DGE and shotgun proteomics.

Overall these analyses assessed 2285 spots/proteins and identified 98 potential markers for skeletal muscle aging in women. These differentially expressed proteins indicate important modifications in cytoplasmic energy metabolism (glycolysis, glycogenolysis), cytosolic-to-mitochondrial NADH shuttle, mitochondrial energy metabolism (Krebs cycle, oxidative phosphorylation pathway) and fatty acid metabolism, which may all be related to dysfunctions in old muscle force generation (Figure 6.1). A fraction of the differentially expressed proteins were linked to myofiber proteins (thick and thin filaments) and to cytoskeletal proteins (microfilaments, microtubules), which may account for alterations in contractile...
properties. Other features support perturbations in cytoprotection and detoxification processes, as we observed differential regulation of several molecular chaperones and of proteins implicated in reactive species detoxification, ion homeostasis and endoplasmic reticulum stress. To the best of our knowledge, the present study represents the most intensive proteomic investigation of skeletal muscle aging in human.

Figure 6.1. Data mining of the expression proteomics analysis

Oxidative and other modifications of proteins occurring during muscle aging and cellular senescence of muscle progenitors

Carbonylated, glycoxidized and HNE-modified proteins were found increased during replicative senescence of human myoblasts. The pattern of the modified proteins was not coincident with the pattern obtained for the total protein staining, indicating that certain proteins represent preferential targets for the different modifications studied. For their identification, proteins derived from total cellular extracts of young (CPD30) and senescent myoblasts were separated by two-dimensional electrophoresis (2D-PAGE). Proteins were identified as targets for either one, two of the three modifications. The identified modified proteins were analyzed for known function and grouped by functional correlations, biological pathways and interaction analyses. Major molecular functions includes several aspects of carbohydrate, amino acids, lipids and nucleic acids metabolism, cellular morphology and assembly, cellular function and maintenance, protein degradation, cell growth and proliferation. Of note, is that enzymes involved in the glycolytic pathway, such as aldolase, GAPDH, PGAM, enolase and pyruvate kinase appear to be quite sensitive to these modifications (Figure 2). The occurrence and characterization of carbonylated proteins was also studied in human biopsies obtained from young and old healthy donors in order to get information about cellular pathways that could be compromised during ageing. An optimized protocol was settled up and validated for the immunodetection of carbonylated proteins after electrophoretic separation of human skeletal muscle proteins derived from rectus abdominis. Although no significant differences in total protein carbonylation between young and old donors was observed at the total proteome level, when the resolution of individual proteins was improved after 2D electrophoresis separation, thirty nine protein spots were evidenced either as increasingly carbonylated or decreased when comparing biopsies from old donors to their young counterparts.

Moreover, protein and protein phosphorylation patterns have also been analyzed both as a function of age and/or upon intervention aimed at counteracting ageing induced sarcopenia such as electro-stimulation training. Indeed, using a functional and proteomic approach, we demonstrated that NMES training is an efficient modality for increasing muscle mass, maximal voluntary strength, neural drive, and oxidative metabolism as well as for improving antioxidant defense systems in healthy young humans. The atypical adaptations of the muscle phenotype by NMES are characteristic of both resistance (i.e. strength gains) and endurance (i.e. fast-to-slow and glycolyticto-oxidative conversion) training and can be mainly ascribed to the specific motor unit recruitment pattern, i.e. nonselective, continuous, and synchronous. The proteomic analysis of elderly subjects pre and post-NMES has been done and have already shown that the MHC isoform distribution pre- and post-NMES undergoing a fast to slow shift in MHC isoforms post-NMES, confirms that NMES stimulation training actually worked.
Post-translational modifications of myofibrillar proteins during aging

A mass spectrometry approach was taken to investigate the effect of aging on myosin post-translational modifications (PTMs) in humans and rats. In humans, type I, IIa and IIx MyHC isoforms gel bands were extracted and screened for acetylation, carboxylation, deamidation, glucosylation, methylation, nitration, ubiquitination, and phosphorylation by LC/MS. Eight PTMs specific to aging were found, among those, 3 were only observed in MyHC IIx isoform while the 5 others occurred in all MyHC isoforms. Two carboxylations were found to be located in the myosin motor domain on the amino acids Pro79, Asn81. The remaining PTMs were situated on the tail region, with carboxylation of Arg908, Asp900/904, deamidation of Asn1168, Gln1164 and methylation of Glu1166. The two modifications found in the motor domain are localized in the Src-homology domain 3 (SH3). While the function of SH3 domain remains unknown, evidence suggests that it facilitates for the myosin essential light chain (ELC) binding to actin and subsequently influences ATPase kinetics and physiological shortening velocity. Thus PTMs in this domain might interfere with ELC and SH3 binding resulting in an impaired power stroke execution.

In rats, type I, IIa, IIb and IIx gel bands from the EDL and Soleus muscles were analyzed from a total of 6 rats, 2 young, 2 middle-aged and 2 old rats by LC/MS. Similar to what was observed in humans, four PTMs specific to aging were identified, among those, one was only observed in the type I MyHC isoform from the soleus while the remaining were not isoform or muscle specific. Half of the PTMs were located in the myosin motor domain (Phe436 oxidation of and Trp438carbonylation); the two others were situated in the tail region (Trp1372 carboxylation and Gln1854 deamidation). The two modifications Phe 436 and Trp 438 (Phe 438 and Trp 440 in the chicken respectively) located in the upper 50-kDa subdomain, are situated near the Switch II loop in a region that is highly flexible and which undergoes multiple conformational changes during the ATPase cycle. Furthermore, those two amino acids are close to several loops or α-helix in particular Phe 246, which is located in the switch II (Fig 6.2). Therefore, such PTMs could lead to the formation of new bonds resulting in an altered flexibility, thus interfering with the ATPase cycle.

Identification of ubiquitin-proteasome and autophagy-lysosome substrates in aging sarcopenia

In order to find the substrate of ubiquitin proteasome we used the knockout mice for Atrogin-1, the muscle-specific ubiquitin-ligase associated to the proteasomal function and muscle atrophy. To address this issue, we analyzed by mass spectrometry the protein accumulated in Atrogin-1 null hearts using SILAC (stable isotope labelling of amino acids in cell culture) mass analysis to determine the relative incorporation of 13C-Lys labeled proteins in heart lysates. Consistent with the reduced efficiency of autophagy/lysosome system in Atrogin-1 KO hearts, metabolic protein labelling, in vivo, with SILAC demonstrated that substrates of lysosomes, including Tau, Filamin C, GAPDH and BAG3, showed decrease protein turnover and increase protein concentration (Figure 3). Of particular interest, CHMP2B (charged multivesicular body protein 2B) which involved in cellular trafficking, was found to accumulate in Atrogin-1 KO mice. Western blotting and immunofluorescence demonstrated that CHMP2B was accumulated in Atrogin-1 KO hearts. We used in vitro experiments to directly test the hypothesis that CHMP2B is degraded by the UPS upon interaction with Atrogin-1. Wild-type neonatal cardiomyocytes were treated with the proteasome inhibitor MG132, which caused CHMP2B poly-ubiquitination and accumulation. In addition CHMP2B co-immunoprecipitated with Atrogin-1 in MG132 treated cells. Atrogin-1 is a ubiquitin ligase belonging to the
SCF family that binds the substrate and forms a complex with Skip1/Cul1/Roc to transfer ubiquitin from the E2 enzyme to the substrate. Importantly, when Atrogin-1 was co-expressed with the Skip1/Cul1/Roc in skeletal muscles, it enhanced CHMP2B ubiquitination. Altogether, these data indicate that Atrogin-1 interacts directly with CHMP2B and mediates its targeting to the UPS for degradation.

In order to find the substrate of autophagy lysosome we used the muscle specific knockout mice for Atg7, the critical enzyme for the autophagosome formation. To address this issue, we analyzed by mass spectrometry the protein accumulated in Atg7 knockout muscle after denervation. The strategy is to highlight the autophagy substrates that should greatly accumulate in denervated. Indeed, in our Atg7 knockout mice the proteasome system is active and therefore, during denervation it induces the breakdown of sarcomeric proteins that constitute the 40% of total myofiber’s proteins. The reduction of sarcomeric proteins better unmasks the proteins that are substrates of autophagy lysosome system. We could identify several proteins that are enriched in autophagy-deficient muscles.

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Figure 6.3. Anomalous protein accumulation in Atrogin-1 knock-out mice. SILAC-based proteomics showed anomalous accumulation of proteins from different cellular compartments in adult Atrogin-1 KO.

Proteasome status in muscle aging and during cellular senescence of human muscle progenitors
One of the main proteolytic systems involved in skeletal muscle protein turnover is the ubiquitin-proteasome system (UPS). Proteasomes composition of muscle biopsy from both healthy subjects and patients with lower limb mobility impairment (LLMI) was analyzed. We found that, despite the expression of β1i and β5i mRNAs, the corresponding proteins of these inducible catalytic subunits were undetectable or barely detectable by western blotting in crude extracts of human vastus lateralis muscle and no age-related modification was observed. Additionally, immunohistochemical analyses on muscle biopsies showed that immunoproteasome subunits were mainly expressed by endothelial cell and infiltrating lymphocytes, if present, and a very faint staining was observed in muscle cells. The analysis of the content of inducible regulatory complex PA28αβ showed similar results. Thus, it seems that the inducible components of proteasomes play a minor role in human muscle ageing and LLMI, whereas a different scenario was observed in other tissues such as the brain (Mishto et al., 2006). Additionally, compared to age-matched healthy subjects, patients affected by LLMI had a higher content of proteasomes, thus suggesting a possible contribution to sarcopenia. Additionally, in these biopsies we also measured proteasomes activity by short fluorogenic peptides and we found an age-related increase of trypsin-like proteasome activity in male patients.

Another part of our study was also focused on myoblasts, the embryonic progenitors cell that differentiate to give rise to muscle cells. Remarkably, we found that PA28αβ regulator and both constitutive and inducible catalytic subunits of proteasome were expressed in myoblast cell cultures from young and elderly healthy subjects. Additional, their expression levels were similar comparing cell cultures at 5 and 21% oxygen tension and no age related modification of proteasomes content was observed.

Proteasome inhibition appears to be significantly correlated with the onset of in vitro cellular senescence in multiple cell types such as fibroblasts and keratinocytes. However, data on senescent human myoblasts has not been reported yet. A significant decrease in chymotrypsin-like, trypsin-like and caspase-like proteasome peptidase activities was also observed in human myoblasts undergoing replicative senescence (starting at 40 population doublings). Analysis of proteasome subunit expression in senescent WI 38 human fibroblasts has previously shown a decreased expression of the catalytic beta subunits explaining the decreased activities observed. However, this is not the case of human myoblasts where
protein levels of the three catalytic subunits remained constant during replicative span and senescence.

The partners/research groups that have been involved in this part of the Myoage project include:
• Bertrand Friguet’s group (University Pierre and Marie Curie, Paris, France)
• Gillian Butler-Browne’s group (Institute of Myology, Paris, France)
• Daniel Béchet’s group (INRA, Theix, France)
• Stephano Schiaffino and Marco Sandri’s groups (VIMM, Padova, Italy)
• Roberto Botinelli’s group (University of Pavia, Italy)
• Lars Larsson’s group (University of Uppsala, Sweden)
• Claudio Franceschi’s group (University of Bologna, Italy)

Work Package 8

Effects of chronic inactivity and subsequent rehabilitation on skeletal muscle mass and function

Deficits in skeletal muscle mass and function are primary outcomes of chronic unloading, with antigravity muscles such as the knee extensors (KE) being particularly affected. An extensive knowledge of these adverse conditions is paramount, for they are inevitably linked to clinical situations such as prolonged best rest or the use of crutches due to bone fracture or musculoskeletal injury. Several models including bed rest, limb cast immobilization and unilateral lower limb suspension (ULLS) have been used to investigate the effects and recovery from disuse. ULLS mimics the standard clinical practice of joint unloading commonly practiced following musculoskeletal injuries or surgery. The disuse-induced loss of muscle strength far exceeds that of muscle mass, indicating the contribution of other factors, such as neural drive, changes in muscle architecture and a decrease in single fibre specific tension. Because muscle architecture is a main determinant of muscle function, changes in fascicle length (Lf) and pennation angle (h) represent powerful indicators of alterations in functional deficit induced by disuse. Pennation, a key-parameter of muscle architecture, is a natural strategy to pack more contractile elements into a given muscle volume. A decrease in pennation angle (h), due to muscle atrophy, is commonly interpreted as a decrease in the number of fascicles in parallel.

In line with the decrease in pennation angle, a reduction in muscle fascicle length implies a loss of sarcomeres in series and a consequential increase in sarcomere excursion during shortening of the whole muscle, shifting the operating muscle length towards a less favourable portion of its length-tension curve. Moreover, a reduction in the number of sarcomere in-series also predicts a decrease in maximum shortening velocity. Hence, beyond gross atrophy, disused muscles are affected by subtle, yet substantial, architectural alterations, resulting in losses of force, velocity and power. Before the completion of the MYOAGE project, information regarding the reversibility of these changes during active recovery was lacking.

Despite the importance of effective recovery strategies in clinical settings, little was known about the resilience of muscle functional properties following periods of disuse.

Hence two of the main objectives of WP8 were to investigate the effects of, a) chronic disuse (unilateral lower limb unloading ULLS) on muscle mass, architecture and function and, b) post-disuse rehabilitation on the recovery of muscle mass, architecture and function.

We hypothesised that substantial remodeling of skeletal muscle architecture would be induced by the 3
weeks of unloading and, unlike passive recovery, a rehabilitation program of equal duration would effectively restore all the muscle structural and functional parameters affected during disuse. The results of the study in terms of muscle strength (MVC), activation capacity (VA), physiological cross-sectional area (PCSA) and force per cross-sectional area (specific tension, SF) before, and the end of the disuse period and after the period of active rehabilitation are summarized in Table 8.1.

Table 8.1
These results show that three weeks of inactivity lead to a 26% loss of muscle strength, corresponding to a rate of loss of 1.24%/day. However, active recovery (strength training) performed three times per week is effecting in fully restoring muscle strength to baseline levels. It is also noteworthy that the loss of muscle strength exceeds that of muscle size, i.e. with inactivity skeletal muscle becomes intrinsically weaker. Again, three weeks of active training fully restores values to pre-disuse levels.

Figure 8.1
When considering muscle volume, it can be seen from Fig 8.1 that 3 weeks of unloading lead to an average loss of quadriceps volume of 12%, with the exception of the bi-articular muscle rectus femoris which, because of its anatomical function, is less prone to atrophy. It is noteworthy to observe that, despite the significant atrophy, quadriceps volume is fully restored within 3 weeks of active rehabilitation.

Figure 8.2
A particularly noteworthy finding of this study was that the chronic unloading on human skeletal muscle induced significant remodeling of muscle architecture in terms of fascicle length (Fig. 8.2a) and pennation angle (Fig.8.2b) which both decreased. These results strongly suggest that disuse muscle atrophy entails both a decrease in sarcomere in parallel (as reflected by a decrease in pennation angle) and in series (as reflected by a decrease in fascicle length). From a clinical point of view this is interesting for it shows that detection of muscle atrophy can be easily be obtained using ultrasound (as opposed to using more expensive scanning devices such as MRI or CT) and that muscle atrophy not only should be viewed from a sheer reduction in muscle thickness (or cross-sectional area) but also as a reduction in the length of muscle fascicles which promptly occurs with unloading.

Biopsy studies on ULLS samples
CSA, Specific force and myosin concentration of muscle fibres
A parallel occurrence of muscle fibre atrophy, loss of specific force and of myosin content after ULLS and a parallel recovery after exercise of all three parameters were observed in both type 1 and 2A muscle fibres. Such findings indicate that the loss in myosin content is a major determinant of the loss in specific force of muscle fibres, which in turn can contribute to the loss of specific force in vivo and that recovery by 3 weeks exercise is complete at single fibre level.

Adaptations in muscle proteome
Down-regulation of troponin and myosin and of several glycolytic enzymes and recovery following exercise were observed. Some antioxidant defence systems were down-regulated (several HSPs and carbonic anhydrase), whereas other were up-regulated (SOD and peroxiredoxin) after ULLS and

Intracellular signalling pathways
No signs of activation of the ubiquitin proteasome system and of autophagy were observed after 3 weeks ULLS suggesting no increased muscle protein breakdown. After retraining, the same pathways were down-regulated compared to pre-ULLS. After 3 weeks ULLS, a lower activation of the Akt/mTOR pathway was observed, which returned to normal following active recovery suggesting lower rate of muscle protein synthesis due to disuse and its normalization following active recovery.

As regards the potential triggers of the above phenomena, PGC1-alpha and NRF2 expression did not significantly change following ULLS or active recovery. Moreover, no protein oxidation and even a decrease of myosin oxidation were observed following ULLS. The latter data suggest no role of metabolism (PGC1-alpha) and redox imbalance (NRF2) in disuse atrophy and recovery notwithstanding the down-regulation of some antioxidant defence systems (Hsps and carbonic anhydrase) observed by proteomic.

Mitochondrial DNA content, mtDNA mutations and muscle transcriptome results on ULLS samples
a) Mitochondrial DNA (mtDNA) copy number and deletion levels
The results showed a trend of an increase for copy number and of a decrease for deletion level after the disuse suggesting a possible compensatory mechanism to preserve the mitochondrial function during atrophy.

b) Mitochondrial DNA content, mtDNA mutations and muscle transcriptome
When comparing the post disuse vs the post rehabilitation samples, a trend for a decrease in copy number and an increase in deletion level. The levels after the rehabilitation are similar to those before starting the disuse period. These data suggested that after a period of training, the levels of mtDNA copy number and deletion could be restored as before the disuse. These results are independent of the amino acid supplementation.

c) Micro RNAs (miRs) profiling (CARD A with 380 miRs by Applied BioSystem)
One of the most interesting results is that miR-132, an important inhibitor of PTEN (phosphatase and tensin homolog), is less expressed both in serum and muscle after 3 weeks of ULLS when compared with pre- ULLS and post training, suggesting a trend of reversibility of the process after training. Similarly, the muscle tissue specific mio-miR-133a decreases after ULLs but tends to recover after training. Further, miR-18a (from the family of miR-17 and involved in TGF- beta signaling and proliferation stimuli) is less expressed in the blood after ULLS and training, while in muscle it increases after ULLS and then recovers after training, thus suggesting that specific miRs have different trends depending on the tissue.

Effects of preoperative raining on post-operative muscle recovery
Older male and female hip surgery patients undergoing preoperative training
Seventeen women aged 46-72 years with late stage knee joint OA scheduled for total knee arthroplasty participated in this study before and after a 2-month home exercise program (HEP) with strengthening, stretching, balance and step exercises. Isometric peak torque (PT) of leg extensors and postural stability characteristics during 30 s standing on a firm or foam surface were recorded. Risk of falls and pain intensity (VAS) were estimated.

The results showed that a significant (p<0.05) increase in PT (Fig. 8.3) and PT:body mass (PT:BM) ratio of the involved leg and bilateral PT and PT:BM ratio was found after the 2-month HEP compared to the data before

Figure 8.3
HEP. PT and PT:BM ratio of the involved leg was significantly (p<0.05) lower compared to the uninvolved leg before HEP. Center of pressure sway length (foam surface) decreased significantly (p<0.05) after HEP. Significant correlations were found between PT of the involved leg and bilateral PT and risk of falls and between PT of the involved leg and postural sway (foam surface) before HEP. Hence, after a two-month HEP leg extensor muscle strength increased and postural sway length on a foam surface decreased. The results indicated that increased leg extensor muscle strength provides improvement of postural stability and diminishes risk of falls in women with late stage knee joint OA.

Aquatic training intervention pre-op patients
Compared with the change in the control group, maximal walking speed increased by 13% (Fig. 8.4) and sit-to-stand time and 8-figure-run time decreased by 15% and 17% in the training group. Training decreased knee pain by 58% compared with controls. Training increased knee extensor power by 30% (p=.016) (Fig. 8.5), and knee flexor power by 30% (p=.021), and also knee flexor torque 66% (p=.031) on the OA side compared with controls. The mean increase in knee flexor power was 37% (p=.010) and in knee flexor torque 15% (p=.038) on the contralateral side compared with the control group. Also, the training increased thigh CSA on OA side by 3% (p=.006) compared with controls. Hence, progressive aquatic resistance training had favourable effects on mobility limitation and on pain of the OA knee. In addition, training increased lower limb muscle power, torque and CSA. Resistance training with additional resistance was feasible mode of preoperative rehabilitation and offered wide-ranging positive effects on patients with late stage knee OA.

Figure. 8.4 Figure.8.5

Effect of life long activity of muscle mass and strength
As opposed to the model of chronic disuse above, WP8 also investigated the results of chronic physical activity in contrasting sarcopenia. This was achieved by studying a group of 23 Master Athletes (MA) aged 68-96 yrs and comparing their morphological and functional characteristics with younger adults (YA), active older (AO) and older frail individuals (FO). The results showed that compared to the young adults, muscle force (F) of the knee extensors was 72% lower in the frail older, 34% lower in the active older but only 22% lower in the master athletes. Differences were smaller but still persisted after normalisation of force to muscle thickness Tm, for F/Tm was 391.0 N/cm in YA, 315.0 N/cm in MA, 302.5 N/cm in AO, 93.9 N/cm in FO, indicating a marked deterioration in muscle quality in the FO and AO, but less in MA. Tm and Lf were lower in the older individuals, particularly so in the FO. However, with increasing age and frailty Tm decreased more than Lf, with a consequential increase in the Lf/Tm ratio (Fig 8.6). This phenomenon was absent in MA, providing additional evidence that the LF/t ratio is a useful marker of sarcopenia. This observation seems of considerable clinical value as it points out that from a single ultrasound scan of the quadriceps muscle (vastus lateralis) it is possible to diagnose sarcopenia. In fact it can be seen in Fig. 8.7 that Interestingly, Lf/t significantly correlated with KE force and appendicular muscle mass evaluated with DXA. Hence we consider that one of the main products of WP8 is that of having identified a new ultrasound based biomarker of sarcopenia represented by the ratio of fascicle length (Lf) to muscle thickness (t); this biomarker is significantly correlated with the conventional DEXA index of sarcopenia (Appendicular...
skeletal muscle mass, ASM, to height squared (ht2).

Figure 8.7

Discordant twins
Twin pairs discordant for physical activity were initially identified from the Finnish Twin Cohort (N=5663 healthy pairs) by the self-reported physical activity assessments done in years 1975 and 1981 (Kujala UM, et al 2002). Overall, 16 same-sex middle-aged and older twin pairs (seven monozygotic (MZ) and nine dizygotic (DZ), five female and 11 male pairs, age range 50-74 yrs) were able to participate to study measurements. The physical activity discordance was defined using a series of structured questions on leisure activity and physical activity during journeys to and from work. Leisure activity was quantified as a metabolic equivalent (average intensity of activity (MET) x average duration of one session (h) x monthly frequency) and expressed as a sum score of leisure time MET hours/day. Maximal isometric left knee extensor force was measured in a sitting position using an adjustable dynamometer chair. T1-weighted MR-images were acquired from left midthigh to analyse total midthigh cross-sectional area, muscle cross sectional area, knee extensor (m. quadriceps femoris) cross-sectional area as well as intramuscular and subcutaneous fat areas.

The results of this comparison showed that the consistently active twins had 20 % higher knee extension forces than their inactive co-twins although the active twins had only 4 % higher midthigh muscle cross-sectional areas. The ratio between the area of midthigh fat and muscle tissues was significantly lower among the active twins.

Work Package 9
Human ageing is accompanied with deterioration in endocrine functions the most notable and well characterized of which being the decrease in the production of sex hormones. Current research literature suggests that low sex hormone concentration may be among the key mechanism for sarcopenia and muscle weakness. However, the underlying biological mechanisms by which age-induced sex hormone deprivation affects muscle mass and function are largely unknown. The main aim of this WP was to investigate the role of estrogens and testosterone in skeletal muscle structure, composition and function among healthy middle-aged and older women and frail older men. In addition, the underlying biological mechanisms for the observed beneficial effects of HRT on skeletal muscle of women were investigated.

This WP utilized multiple study designs including women and men 50 years and older. Studies are: 1) 1-year randomized double-blinded, placebo-controlled trial (RCT HRT-STUDY) including 50–57-year-old postmenopausal women randomized into four groups; HRT, plyometric jumping exercise (Ex), ExHRT, and controls (Co). The HRT was a combined estradiol (2 mg) and norethisterone acetate (1 mg) product (Kliogest; Novo Nordic). Exercise included progressive high-impact jumping activities (Pöllänen et al. AGE 2010). 2) A case–control HRT discordant study with monozygotic postmenopausal 54–62-year-old female twins (TWIN STUDY) included 15 postmenopausal twin pairs of whom one co-twin was a current HRT user (mean duration 7 ± 4 years) and the other co-twin had never used HRT. (Ronkainen et al. J Appl Physiol 2009). 3) Cross-sectional ERT-STUDY included 10 61 ± 4 years old hysterectomized/oophorectomized women who were using estrogen replacement therapy (ERT, daily 2 mg oral 17β-estradiol) and 10 age-matched postmenopausal controls (60 ± 4 years) (Hansen et al 2012).
4) A randomized controlled cross-over study (TRANSDERMAL-ERT study) included eleven postmenopausal women (65 ± 2 years) who went through a 5 day control period and a 5 day period, where two transdermal ERT patches were placed on the skin (Evorel©50 lg/24 h estradiol). At day two of each period, the participants performed a strenuous one-legged resistance exercise bout to study the interaction between an enhanced estrogen level and exercise (Pingel et al. J Appl Physiol 2012). 5) Randomized, double-blinded, placebo-controlled trial (RCT T-STUDY) included 30 men aged 65 years and older with one or more frailty criteria with low endogenous serum (less than 12 nmol/L) or calculated free testosterone levels (250 pmol/L). Testosterone group received a transdermal hydroalcoholic testosterone gel (Testogel 1%; Bayer Schering Plough) at a dose of 50 mg/day for 6 months. The dose was adjusted to 75 or 25 mg/day according to serum testosterone at day 10 and 3 months. The control group received a matched placebo gel. (Atkinson et al J Gerontol Bio Sci 2010).

RCT HRT-STUDY and TWIN STUDY showed that estrogen containing HRT is beneficial for muscle function, especially when the activity is performed with fast muscle contractions. We observe 14–16% greater muscle power, 32% greater plantar flexor twitch torque and 7% greater walking speed among HRT users compared to non-users. In addition, we observed 7% greater thigh muscle cross-sectional area after 1-year HRT compared to the controls not on hormonal therapy. Women, who had been on HRT for an average of 7 years, had 8% greater relative thigh muscle area and an average 5% smaller relative fat area in the thigh compared to the non-users. HRT was not associated with mean muscle fiber cross-sectional area of muscle fiber composition. As expected, plyometric training for one year increased muscle power. In addition, a significant increase in lean body mass was observed together with increased thigh muscle attenuation coefficient compared to the controls. Exercise in combination with HRT exceeded the benefits of Ex or HRT alone. HRT is beneficial for muscle function and it preserves muscle mass and prevents fat infiltration into the muscle. (Finni et al. Muscle Nerve 2011, Ronkainen et al. J Appl Physiol 2009).

The effects of estrogen replacement therapy on skeletal muscle protein synthesis rates were investigated in ERT-STUDY. Results showed a significant increase in myofibrillar fractional protein synthesis rate in response to resistance exercise in the ERT users. No difference between the exercised and contra-lateral leg was observed in the controls. The data support a synergistic anabolic effect by estrogen administration combined with strenuous training. (Hansen et al. J Gerontol A Biol Sci Med Sci 2012)

As we observed that estrogen containing hormonal treatment had significant contribution to the skeletal muscle function and performance of postmenopausal women, a detailed contractile protein analysis was performed at the single fiber level from the muscle biopsy samples. The results showed that HRT use was associated with a significantly smaller (~27%; P < 0.05) mean myonuclear domain size in muscle fibres expressing the type I but not the Ila myosin heavy chain (MyHC) isoform. In comparison to HRT non-using twins, higher specific force was recorded in HRT using co-twins both in muscle fibres expressing type I (~27%; P < 0.05) and type Ila (~23%; P < 0.05) MyHC isoforms. These differences were fibre-type dependent, i.e. the higher specific force in fast-twitch muscle fibres was primarily caused by higher force per cross-bridge while slow-twitch fibres relied on both a higher number and force per cross-bridge. HRT use had no effect on fibre cross-sectional area (CSA), velocity of unloaded shortening (V0) and relative proportion of MyHC isoforms. Since the two prime determinants of specific force are the fraction of strongly attached cross-bridges and the force produced by individual cross-bridges, the results from this study suggest that both factors contribute to the higher specific force in HRT users, but the relative contribution appears to be MyHC dependent. Furthermore, HRT had a significant effect on the myonuclear
In order to comprehensively explore the genome-wide activities in skeletal muscles of postmenopausal women, we carried out microarray analyses for the whole transcriptome from the muscle samples. Our pioneering RCT STUDY showed that at the early stage of postmenopause, skeletal muscle gene expression changes notably (4195 transcripts) and that a yearlong HRT effectively reduces these changes (97 transcripts) (Pöllänen et al. Rejuvenation Res 2007). Long-term HRT use (TWIN STUDY) was associated with higher expression of 22 genes and lower expression of 33 genes. Of these, genes related to organization of the cytoskeleton, cell-environment interactions, energy metabolism and responses to nutrition explained a substantial fraction of the observed variation in muscle composition (18-26%) and power (19-20%). Although energy metabolism was affected through downregulation of the transcripts related to succinate dehydrogenase complex in mitochondria, no differences were observed in number of mitochondria or oxidative capacity per muscle sample cross-section (Ronkainen et al. Aging Cell 2010).

Muscle tissue is regulated by many overlapping and interacting signalling cascades. IGF-1/PI3K/AKT pathway activates protein synthesis and inhibits degradation thereby controlling the net balance of muscle protein turnover. The IGF-1 pathway can be activated by e.g. muscle contraction, insulin, IGF-1, and sex steroids. The TWIN-STUDY showed that the skeletal muscle of postmenopausal women not on HRT had significantly lower expression of IGF-1 compared to the premenopausal women at age 30 to 40 years (28% for IGF-1Ea and 40% for IGF-1Ec, i.e. mechano growth factor, MGF). In addition, twin sisters using HRT had 28% higher expression of IGF-receptor than their co-twins not on HRT. (Ahtiainen et al. AGE 2012). RT for one year (RCT HRT-STUDY) increased significantly the intramuscular expression of IGF-1 gene (13%) compared to the placebo-treated control group (-16%). Of the other genes along the IGF-1 signalling pathway, HRT down regulated AKT1, up regulated AR and IGF-1Ec (HRT 58%, Co -31%, Fig 9.1) and maintained FOXO3 and mTOR expressions compared to the control group. (Pöllänen et al. Growth Hormone IGF Res 2010).

Proinflammatory cytokines, such as IL6, are known to act as negative regulators of IGF-1. They control the secretion of IGF-1, decrease IGF-1 sensitivity by increasing the production of IGF binding proteins, and they can cause IGF-1 resistance. In the TWIN-STUDY, a strong negative association (r = -0.71 P=0.01) was found between serum IGF-1 and serum IL6-R in HRT using postmenopausal women, but not in their non-using co-twins. It can be concluded that in postmenopausal women IGF-1 resistance in skeletal muscle may be associated with the inflammatory status (Ahtiainen et al. AGE 2012). HRT potentially resists the enhancement of inflammation by increasing the amount of IGF-1R and by stimulating the post-receptor signalling. However, we cannot distinguish the direct effects of HRT on the IGF-1 pathway from possible indirect effects via e.g. IL-6 signalling. Altogether, these data suggest that HRT may have positive anti-catabolic effects on ageing skeletal muscle of women.

Transdermal estrogen patches do not effect liver metabolism and change the level of circulating IGF-I similarly as oral ERT and HRT. Therefore, to study the effect of estrogen administration on skeletal muscle independent of effects induced by changes in circulating IGF-I, an experiment utilizing transdermal
estrogen treatment was performed. If muscle fibre area is increased by ERT and training, a remodelling of the surrounding muscle connective tissue is required. Accordingly, the TRANSDERMAL-ERT study showed that ERT combined with resistance exercise enhanced the synthesis of collagen (PINP) within muscle connective tissue (interaction p<0.01). Furthermore, in the ERT-STUDY a higher tendon collagen synthesis rate, changes in the fibril characteristics and lower relative tendon stiffness were observed in the ERT-users compared to controls, and a positive correlation between tendon collagen synthesis rate and the circulating estradiol was observed among ERT-users. (Hansen et al. J Appl Physiol 2009, Hansen et al. Scand J Med Sci Sport 2012, Pingel et al. J Appl Physiol 2012).

Although male andropause is not as clearly defined as female menopause, it includes a gradual decrease in androgen production leading to a wide variety of symptoms occurring in men after middle age. A growing number of studies have specifically assessed whether testosterone therapy can partly reverse the ageing-related effects of reduced muscle mass and strength, in an effort to discern whether this intervention can be employed in clinical practice for this purpose. RCT T-STUDY showed that transdermal hydroalcoholic testosterone gel resulted in a preservation of muscle thickness at 6 months while it decreased in the placebo group (effect size 1.4 mm, CI95% 0.3 to 2.5 mm; P=0.015). There was no significant effect of treatment on fascicle length (effect size 1.9 mm CI95% −1.2 to 5.0 mm; P=0.22) or pennation angle (effect size 1.2° CI95% −1.3 to 3.7°; P=0.32). Despite a 5% increase in plantar flexor strength in the T group and a decrease of 12% in the placebo group, no significant effect of T was observed. (Atkinson et al J Gerontol Med Sci 2010).

To summarize, estrogen containing hormonal therapy in postmenopausal women is shown to diminish age-associated muscle loss, loss in fast muscle function, and accumulation of fat in skeletal muscle (Fig 9.2). Further, estrogen raises the protein synthesis rate in skeletal muscle after resistance training, and has an anabolic effect upon connective tissue in both skeletal muscle and tendon, which influences matrix structure and mechanical properties. Significant positive effects on regulation of muscle contraction and myonuclear organization were observed at the cellular level in response to hormonal therapy in fiber type dependent manner. IGF-1/PI3K/AKT pathway is among the biological mechanisms underlying the observed effects of hormonal therapy in skeletal muscle. In addition, among frail older men, testosterone therapy mitigates sarcopenia by improving skeletal muscle tissue. (Sipilä et al Biogerontology 2013).

Figure 9.2 Contribution of HRT on skeletal muscle function, composition and quality in postmenopausal women.
The studies involved the following partners and their research groups: professor Sarianna Sipilä and laboratory chief Vuokko Kovanen, University of Jyväskylä, Finland; Professor Michael Kjaer Institute of Sport Medicine, Copenhagen, Denmark; professor Marco Narici, Manchester Metropolitan University, United Kingdom; Professor Lars Larsson University of Uppsala; and professor Gillian Butler-Browne, Institut National de la Santé et de la Recherche Médicale, Paris, France.

Work Package 10
The objective of WP10 was to update the knowledge on ethical requirements toward recruitment of older research subjects, obtaining of informed consent, collection of biological samples, and use of stem cells in preclinical and clinical settings. The studies involved the following leading Partners:
The ethical issues have been dealt with during the meeting of WP leaders in Bologna, on February 22, 2010 and at annual meeting in Padova on October 27-30, 2010. The standard operation procedures applied for collecting blood and muscle specimens and cultivation of muscle cells have been thoroughly reviewed in terms of their relevance to ethical requirements. The conclusion is that the SOPs applied ensure uniformed quality of samples in all phases - taking, storing and transferring to Partners. The number of subjects to be studied and their inclusion-exclusion criteria have been agreed between the Partners, whereas the recruitment of subjects has been a routinely discussed issue. The major problem is that a significant share of the subjects participating in the study has not been willing to donate transcutaneous muscle biopsy, so the Partners have agreed that the original research plan can be modified without compromising the scientific core of the project with fewer samples collected. Feedback from the subjects participating in the study has been continuously monitored in research groups. The Partners have been informed on a regular basis concerning recent knowledge in the field of ethics of aging research during both abovementioned meetings. In co-work with Partners 6 and 16, the manuscript "Ethical aspects of human aging research: an update" has been published in Biogerontology (Seppet et al, 2011). The review updates the knowledge in ethical requirements regarding the recruitment of older research subjects, obtaining of informed consent, collection of biological samples, and use of stem cells in preclinical and clinical settings. It is concluded that the application of adequate ethical platform markedly facilitates the recruitment of older persons for participation in research. In order to guarantee successful research on problems of human aging, protect older people from undesired interference, and afford their benefits through supporting innovations in research, therapy and care, the related basic ethical concepts are currently extensively discussed, with all interested parties participating.

The possibilities of harmonization of the cell culture protocols (media, e.g. with serum vs without serum, cytokine doses, use of purification by MACS CD56 technology) between the WP5 and WP3 have been discussed, recently during annual meeting in Jyväskylä in October 2011. A current consensus has been reached concerning different laboratories using culture methods that vary depending on the task and aim of the studies. Therefore it is impractical or even impossible to strictly standardize the protocols of cell culture methods. At the same time it is reasonable and possible to compare the data obtained with traditional methods of cell cultivation with those obtained from the cells purified by application of MACS CD56 technology.

It has been agreed that the related databases could be used by Partners after official termination the MYOAGE project, this approach promotes continuation and sustainability of the established scientific co-operation between the Partners.

The following standardized operational procedures have been elaborated, tested and implemented:
• Tests for assessment of thigh muscle strength and fatigability have been elaborated and standardized among the partners.
• Methods for assessment of subjects’ physical activity have been elaborated and standardized among the partners.
• A program of resistance exercises and 2-month diary protocol for self-assessment have been elaborated by physiotherapy specialist and researcher. Patients receive THERA bands with explanation of their application and illustrated description of exercises. The diary for self-control addresses reporting on pain, fatigue, sleeping and exercise performance.
• A method of purification of myoblasts by using MACS CD56 tecnology has been harmonized between
the laboratories in Paris (Dr. Gillian Butler-Browne’s group, Partner 1) and in Tartu (Dr. Enn Seppet’s group).

**Work Package 7**

Among MYOAGE objectives, training and formation of the junior scientists were two important issues. For this purpose, MYOAGE organised 4 international workshops to address several hot-topics of the ageing muscle research.

**Figure 7.1** The 1st periodic Workshop was organised in Split from September 22nd to 25th, 2010.

**Figure 7.2** The 2nd periodic Workshop on Muscle Mass Regulation was organized in Acaya, near Lecce, Italy from September 23rd to 25th, 2011.

**Figure 7.3** The 3rd periodic Workshop on Inflammation and ECM remodelling in Ageing was organised in Barcelona, Spain from December 1st to 2nd, 2011.

**Figure 7.4** Last workshop in the serie, the 4th periodic Workshop entitled The aging human muscle: An integrated machinery was organised in Copenhagen, Denmark from August 30th to 31st, 2012.

The feedback on these 4 events was evaluated and showed their very high scientific quality. Overall, these workshops gave the opportunity to junior scientists including PhD students to present their work and freely interact with the speakers and expert scientists present, setting the bases of future interactions in the field.

In term of dissemination of the results, it was also of utmost importance to MYOAGE to present its results as a whole to a larger scientific community. This was done when the workpackage leaders presented their most prominent results at the European Muscle Conference, in Rhodes on September 1st to 5th 2012.

**Figure 7.5**
A- Prof. Stefano Schiaffino (VIMM) giving the opening keynote presentation to the EMC participants
B- Dr. Gillian Butler-Browne (Inserm) and Prof. Marco Narici (MMU) chairing the muscle aging session
C- Dr. Gillian Butler-Browne opening the ageing muscle session at the EMC
D- the MYOAGE WP leaders ready to answer the questions following their presentations. From left to right: Pr. Claudio Franceschi, Pr. Marco Sandri, Pr. Sarianna Sipilä, Pr. Roberto Bottinelli, Pr. Antonio Musaro and Pr. Bertrand Friguet.
E- Group photo of the EMC 2012 Participants including MYOAGE members.

Last but not least, the joint dissemination actions included the publication of no less than 9 papers covering all aspects of the MYOAGE research in a single issue of Biogerontology June, 14th 2013. This joint publication demonstrates the excellent interaction and collaboration which took place within MYOAGE and the ability of its members to achieve important progress in understanding and combating the age-related muscle weakness.

**Work Package 11**

MYOAGE management allowed the project to run smoothly in the best collaborative conditions. The
manager brought a daily support to the project implementation and organized the contractual meetings. Only 2 grant agreement amendments were necessary (one partner change and a 6-month extension) during the course of the project and the close monitoring of the budget allowed all partners to do their share of the work without problem. From this point of view also, MYOAGE was a successful project.

Potential Impact:
Within Myoage we have constituted one of the largest biobanks of human blood and muscle biopsies from well-characterized individuals of different ages. The combination of the clinical dataset and the biological dataset empowers the consortium to provide unique insights into muscle ageing leading to strategies to combat age related muscle loss and weakness. We have established a unique dataset providing ‘normative, reference’ values to describe healthy ageing in a European Population. This multi-disciplinary pan-European approach has allowed us to define healthy and impaired muscle ageing and to identify new biomarkers and risk factors of muscle weakness in older age. The dataset will form the basis from which new hypotheses and investigations can develop. We have raised awareness of the extent of muscle loss and weakness that occurs even during ‘healthy’ ageing. Of high impact and importance, we have developed new techniques and tools to quantify the loss of muscle with ageing and shown that considerably more people have ‘sarcopenia’ than previously thought. The biobank and databases are the framework to further explore pathways and strategies to define tailored therapies against sarcopenia for our growing number of elderly EU citizens. Our final aim is to define the various parameters that characterise healthy muscle ageing with a level of mobility that does not hamper independent normal life, as opposed to muscle frailty and weakness. It is interesting to note that particular attention is being given to cellular and molecular mechanisms that could be potential targets to counteract muscle weakness and frailty. Results have already shown that some obvious targets needed to be further investigated, since gross modifications of these pathways were deleterious to muscle function. One good example of this is the massif age associated increase in post-transcriptional modifications of the skeletal muscle proteome. Another striking theme to emerge from the integration of results from this project was that findings from animal models are not necessarily an indication of human ageing. Detailed examination of human tissue offered several “surprise” findings and emphasized the role of an “ageing environment” on muscle and stem cell function and regenerative capacity. We have dissected of the signal transduction networks that control muscle fiber size, and have identified novel, unexpected players, such as the Bone Morphogenetic Proteins (BMPs). A major result concerns the role of autophagy in the maintenance of muscle homeostasis. While the efficiency of autophagy declines during ageing thus leading to the accumulation of intracellular waste products, exercise training can counteract this process. These results raise a number of exciting questions, which are now the focus of active research in many labs: Can boosted autophagy explain the life-extending effects of exercise? Can changes in skeletal muscle affect the whole organism? Can one devise new approaches to treat age-related degenerative diseases by manipulating autophagy? The inflammatory status of old people has found to be correlated with muscle strength, thus highlighting the connection between inflammation in old people (inflammaging) and sarcopenia, as well as new possible non-invasive biomarkers of sarcopenia. In addition physical exercise not only maintains muscle mass and strength but also decreases the level of these inflammatory mediators. We have identified changes in muscle metabolism that are shared by old subjects and young inactive patients, suggesting that physical inactivity to a certain extent mimics muscle ageing. Many of the discoveries that have been made by the MYOAGE consortium could eventually become new guidelines to improve healthy aging. In addition to exercise which is the golden standard to combat age related muscle weakness but for which
we need to determine exactly what type of exercise we should prescribe we have also demonstrated the beneficial effect of preoperative training, the very positive beneficial effect of hormone replacement therapy as well as the central role played by the IGF1 pathway pinpointing this not only as a good biomarker but also as an ideal therapeutic target. We hope in the future that business, industry and the third-sector can exploit our research findings by promoting the aspects we have identified as ‘healthy ageing’ e.g. through provision of exercise, nutrition or lifestyle interventions (provision of personalised prescription, recording of activities and rehabilitation of individuals at high risk of falling) for the over 60s.

One important impact of the network will be the decrease of costs associated to the increase of life expectancy in Europe: the guidelines generated, based upon functional evaluation of the muscle potential of the population will provide a specific framework to help the generation of decisions at a European level regarding the ageing population, such as the retirement age and conditions or the specific tools to be developed to ensure healthy ageing.

Finally, the MYOAGE consortium has increased individual awareness of muscle wasting and weakness in older age and promoted the initiative of “active” ageing to maintain muscle strength and healthy ageing through public engagement and other dissemination events.

List of Websites:

www.myoage.eu

Related documents

Jay final1-figures-final-report.pdf

Last update: 12 December 2014
Record number: 153872

Permalink: https://cordis.europa.eu/project/id/223576/reporting

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