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THE ELDERLY HEALTH STATUS AND ITS CORRELATION WITH AGEING BIOMARKERS: THE EUROPEAN PROJECT MARK-AGE

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1. INTRODUCTION

Since the mid-nineteenth century approximately, it's began an unstoppable demographic revolution that in less than two centuries has led the life expectancy from about 40 to beyond the current 80 years, in other words a gain of about three months per year over the past 160 years (Oeppen & Vaupel, 2002). This extraordinary lifespan increase is so widely spread to affect not only the most economically developed countries, but involves also other demographic giants as China and India and therefore should be considered a global phenomenon.

According to the latest statistics projections formulated by Eurostat, the proportion of elderly EU-27's population aged over 65 years old is predicted to increase from 17.5 % in 2011 to 29.5 % by 2060 while the number of individuals aged 80 years and beyond is predicted to triplicate between 2011 and 2060 (Eurostat source, Capri et al. 2014).

This "population explosion" makes extremely important to identify the factors (biological and non-biological) involved in aging devoid of major diseases and disabilities, identify the different genetic and molecular mechanisms which underpin the morbidity and mortality along with new strategies able to counteract or slow down its progress and in particular to decelerate the onset of age-related diseases and disabilities in order to increase the number of elderly people in good health.

In this scenario the need of biomarkers of healthy or unhealthy ageing seems to be more and more necessary and urgent.

The European Project MARK-AGE (European Study to Establish Biomarkers of Human Ageing) attempts to provide an answer to these needs that represent a strategic priority of the European Community in the context of health policies. The expected results will contribute to a greater understanding of the biological mechanisms of human ageing contributing to develop focused prevention strategies and to influence the diseases process and above all to improve their therapy.

1.1 HUMAN AGEING

1.1.1 Demographic Ageing

The aging of the population is currently one of the most significant worldwide demographic events (with substantial different timing and dynamics across countries), where it is producing the transformation of economic, social and cultural development and it will intensify during the twenty-first century. (Schoeni & Ofstedal 2010, United Nations 2013). In developing countries population ageing is a fairly new phenomenon but considering the rate of growth of their older population segment (significantly higher than in developed countries) it is reasonably expected that the older population of the world will increasingly be concentrated in the less developed regions.

Which are the causes of this unprecedented phenomenon? Ageing is a dynamic process and many factors had contributed to the increase at global level of population aged 60+ and 80+ (Bloom et al. 2011): the decrease of mortality, the increasing life expectancy and fertility rates that in recent decades have reduced the relative number of young people and pushed up the share of the elderly. In support of this assertion there are data from **World Population Prospects: the 2012 Revision** (United Nations 2013), according to which the decline of fertility rate in most countries of the world in the last decades has been the main factor driving population ageing.

According to Eurostat the consistently low birth rates and higher life expectancy will transform the shape of the EU-27's age pyramid (Fig.1) and most likely the most significant change will be the marked transition towards a much older population structure and this development is already becoming apparent in several EU Member States (Eurostat 2012).

The demographic aging of the population is a long-lasting process, because in the course of the twentieth century, the proportion of older people has continued to grow much faster than the growth of the total population. Within the elderly population, the age group that seems to grow more rapidly in the world is represented by people aged 80 years or more (Fig. 1).

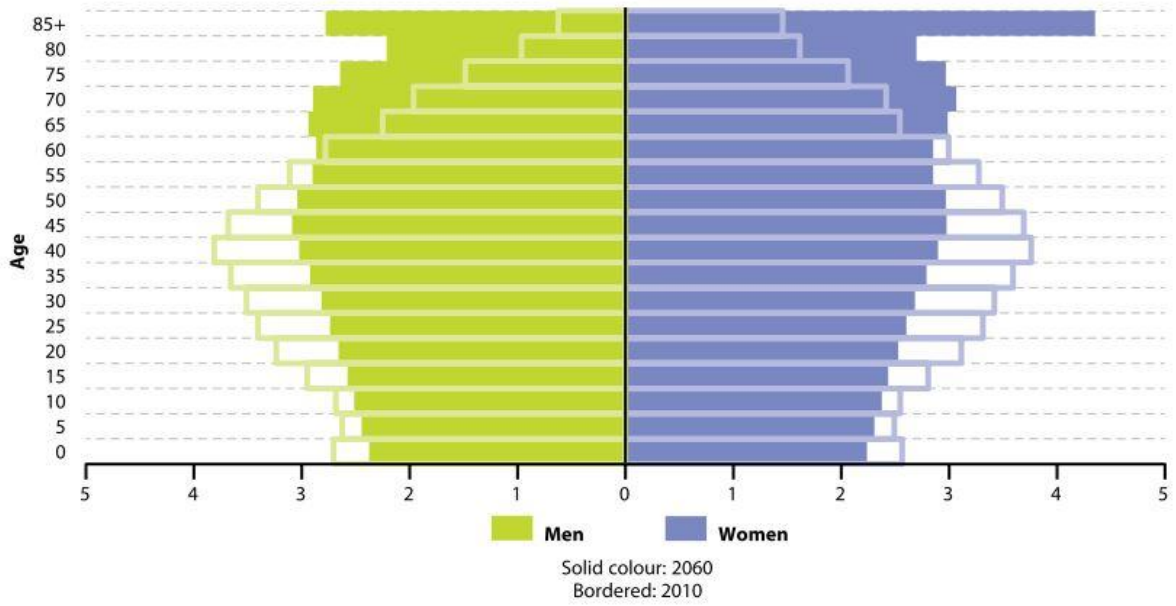


Fig. 1Population pyramids, EU-27, 2010 and 2060 (% of the total population): 2010, provisional; 2060 data are projections (EUROPOP2010 convergence scenario). Source: Eurostat

In order to fully understand what implications this phenomenon may have it is important to take account of two significant demographic indexes: the ageing index and the old-age-dependency ratio index.

The ageing index represents a dynamic indicator that estimates the degree of population aging. The ageing index is a composite demographic ratio, defined as the percentage between the old age population (over 65) and the young population (under 15). It is one of the several demographic indicators that can be used to measure the rate at which a population ages. Values exceeding 100 indicate a greater presence of elderly subjects compared to young (Fig. 2).

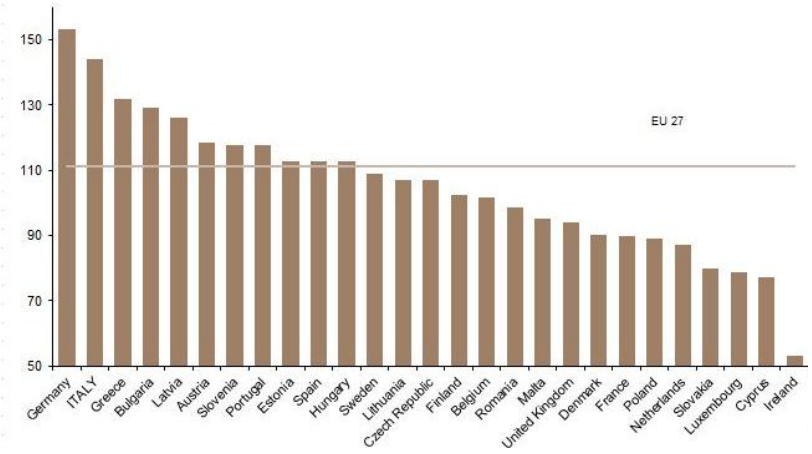


Fig. 2 Ageing index in EU countries - Year 2010 (percentage values) Source: Eurostat

According to **World Population Ageing 1950-2050** (United Nations 2010), by 2050, the ageing index is predicted to range from a high of 263 per hundred in Europe against a low of

37 per hundred in Africa (Fig. 3). This means that by the end of the first half of this century, there will be almost three persons aged 60 or over for every child under 15 years in Europe, and almost three children under 15 years for every person aged 60 or over in Africa.

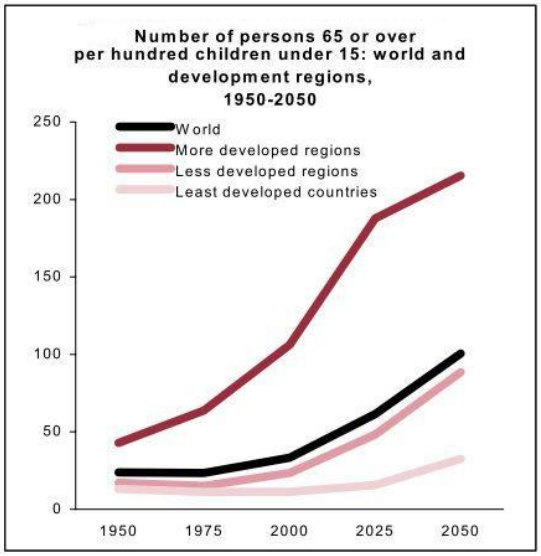


Fig. 3 Ageing index projections 1950-2050 (Source United Nation 2010)

The old-age dependency ratio demonstrates the proportion of the population aged 65+ relative to the working-age population. Dependency ratio is able to provide only an approximation of the dependency burden. In fact not all the elderly require support and on the contrary, there is evidence that older persons in many societies are providers of support to their adult children (Morgan, Schuster and Butler, 1991; Saad, 2001). As for ageing index, the old-age dependency ratio projections for 2050 are less than encouraging (Fig. 4):

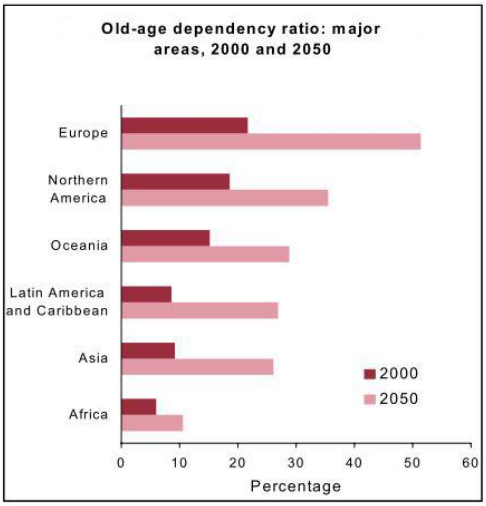


Fig. 4 Old-age dependency ratio projections 1950-2050 (Source United Nation 2010)

United Nations estimates that the old-age dependency ratio will almost double in Northern America, Africa and Oceania; it will more than double in Europe; and more than triple in Asia and in Latin America and the Caribbean (Fig. 4)

Due to the fact that old-age dependency ratio is considered to be a coarse index it has been proposed to decompose it into two additive indicators: **the old- age unhealthy dependency ratio** and **the old-age healthy dependency ratio**. The new indicators distinguish between a) the burden of those who are in good health and could potentially remain in the labour force; and b) those who are disabled or have chronic conditions and are more likely to require financial and health care support (Muszyńska & Rau 2012).

With this premises a real emergency, related to the strong growth of the great elders who in all likelihood be accompanied by a dramatic decline of people with less than 80 years, can be expected. Another important feature of the demographic trend is the greater number of older women than men that make up over 70% of the individuals aged over 80 years. All these factors are crucial in terms of health and social care, because octogenarians women represent the portion of the population with the greatest care needs, given the high degree of co-morbidity and disability that characterizes them (Gollini 2005).

1.1.2 Social and medical consequences of ageing

Aging population is a phenomenon that affects the entire world and that may have important implications in the fields of social and health services. Aging does not involve only the elderly but also families, as it grows the number of coexisting generations, and the general population since we have an increase in the proportion of elderly and old people in the total population along with a contraption of births.

While the strong demographic aging is the manifestation of significant medical, social, and economic advances over disease that is able to lengthen the life expectancy, on the other hand it also presents tremendous challenges since it leads to a greater number of age-related diseases and bring out the question of long term assistance.

The response to this population needs is measured through home care and specialized institutions. This is therefore an issue that can become difficult to sustain by the society, if not

accompanied by appropriate interventions. It must be remembered that age brings double the risk of comorbidity and disability, and the fragility of the elderly is characterized by this double jeopardy and the psycho-social failure that comes with it. One of the important points in the analysis of an aging population it is the disability and loss of functional autonomy, which unfortunately often characterized the last years life.

This rapid aging of the population is associated with major changes in the structure and roles of family such as the vertical of the extended family, the rise of single-person households and single women and widows. This consequently leads to the necessity of help for those who have lost autonomy in daily life along with problems of intergenerational solidarity (also due to the different social roles of women, traditional lenders of care) and long term care. The lack of social and family relationships that more and more frequently characterize the elderly are causal factors of mortality and morbidity (Keller et al., 2003). Generally, loneliness in the elderly becomes a cause of suffering and psycho-physic regression. The older couples tend to isolate sometimes more than single subjects. Finding new strategies that can allow to better address this new "trend" demographic it's extremely necessary. From the Assembly of Madrid, the World Health Organization has implemented a series of policies and programs that aim to promote what has been named "Active Aging", which has as its objectives the autonomy, the independence, quality of life of the individual, taking into account that mental health and social relationships are critical to the improvement of the physical state.

European Union took note of the necessity to reform and adapt social protection systems, including health care, to cope with an aging population and identified three key point:

1. **Accessibility of care** which must take into account the needs and difficulties of the most disadvantaged groups and individuals, but also to those who need costly long-term care;

2. **Offering quality care** that keeps pace with scientific research and that adapts to the needs associated with the aging process and based on an evaluation of health benefits;

3. **Long-term financial sustainability of care** and a more efficient system.

1.1.3 Ageing mechanisms

Aging is a complex process not entirely understood. Aging is a process that affects most of living organisms. In humans from the moment of conception until death occur a series of changes in processes and physiological parameters, with the acquisition of new skills and loss or maintenance of others, which determines the resulting aging of the whole organism. During the last decades many theories were formulated, one of the most recent is that proposed by MV Blagosklonny (Blagosklonny 2013), named "quasi-programmed", where aging is a wasteful and aimless continuation of developmental growth, driven by nutrient-sensing, growth-promoting signaling pathways such as MTOR (mechanistic target of rapamycin) (Fig. 5, 6 and 7).

Table 1. Comparison of 3 groups of theories of aging: programmed, stochastic, and quasi-programmed

Theories	Defining feature	Purposeful?	Programmed?	Caused by ROS?	Kills via age-related diseases?	Causes death directly?	Menopause in women is	Link between aging and diseases	Use of energetic resources
Programmed	functional decline	yes	yes	mostly	unspecified	yes	programmed	unspecified	unspecified
Stochastic	functional decline	sometimes*	in some cases*	mostly	sometimes#	yes	programmed	vulnerability to diseases#	slows aging (via repair)
Quasi-programmed	hyperfunction	no	no	no	always	no	prototypical disease	manifested by diseases	fuels aging (via TOR)

According to stochastic theories, aging is caused by random accumulation of damages, errors, and "garbage" due to multiple causes including but not limited to free radicals. *Stochastic theories still accept that aging can be purposefully programmed (e.g., in salmon). #According to stochastic theories, aging can kill directly (by non-specified mechanisms) and also increases the vulnerability to age-related diseases.

Fig. 5 Ageing theories comparison (Blagosklonny 2013)

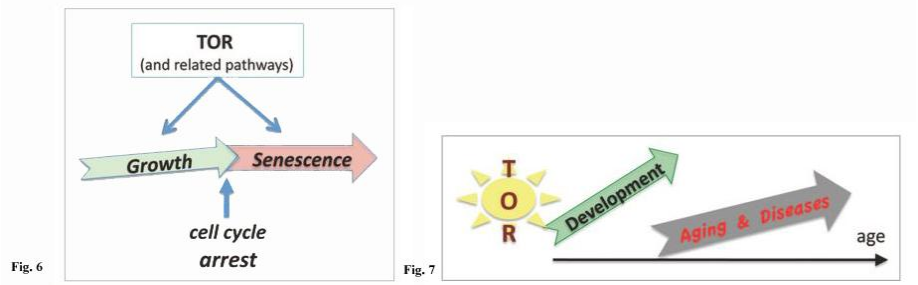


Fig. 6 From cellular growth to hypertrophic senescence (gerocon-version) (Blagosklonny 2013).

Fig. 7 From developmental growth (program) to aging (shadow) (Blagosklonny 2013).

Ageing is well known to be characterized by considerable heterogeneity due to the complex interactions between genetics and epigenetics, affecting the rate of ageing in humans (Capri et

al., 2006; 2013), environmental conditions (Biagi et al., 2013; Garm et al., 2013) and stochasticity.

Aging has been described as a complex mosaic, resulting from the interaction of a variety of environmental, stochastic and genetic-epigenetic events/stimuli and affecting differently all levels of biological organization (macromolecules, organelles, organs etc.). (Cevenini et al., 2008) (Fig. 8) According to this mosaic depiction, tissues and organs might age at different rate and further, individuals of same chronological age might have different biological age (Capri et al., 2014). The presence of this complexity surely makes more difficult the identification of a unique comprehensive mechanism of ageing and related biomarkers (Capri et al., 2014, Deelen et al., 2013).

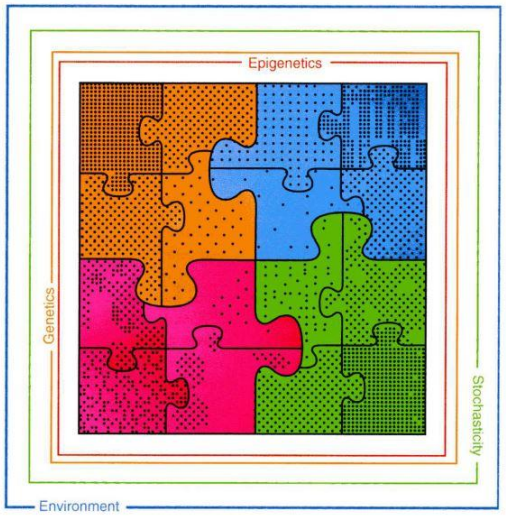


Fig. 8 Representation of ageing mosaic in which every organ (represented in different colour) is differently affected in various degree. The frame (in various colours) indicates the effect of genetics, environment and stocasticity (Cevenini et al. 2008)

The phenotype of the aged individuals is very complex because is the result of the continuous remodelling ability of the body (Franceschi et al., 2000; Spazzafumo et al., 2011) to adapt to the unrepaired damages which can occur to the level of macromolecules, cell, and organs. According to the remodelling theory of aging (Franceschi et al. 1995, 2000;) the ageing phenotype is the result of two major process:

• accumulation of unrepaired damages at all levels of the biological organization (molecules, cells, tissues, organs)

• capability of the body to react/adapt/neutralize such damages by activating a variety of physiological molecular and cellular repair and defence responses/pathways

The remodeling that occurs in the human body is based on and performed by fundamental biological responses, such as DNA repair, apoptosis, immune response and inflammation "(Franceschi 1995, 2000). This process can take different pathways during the different phases of life. In fact it represents an extremely positive factor, in terms of survival, during the reproductive phase of life but it may be detrimental in the post-reproductive period of life since this was unpredicted by evolution (antagonistic pleiotropy) (Franceschi 2000). So a successful remodeling can lead to longevity while on the contrary an unsuccessful remodeling can take the body to disease and death.

One of the key point of aging process is inflammation in fact ageing is accompanied by a low grade of chronic inflammatory status derived from an imbalance between inflammatory and anti-inflammatory networks (Franceschi et al. 2000, 2007) (fig. 9). This phenomenon was denominate "Inflammaging (Franceschi et al. 2000, 2007, Cevenini et al., 2013).

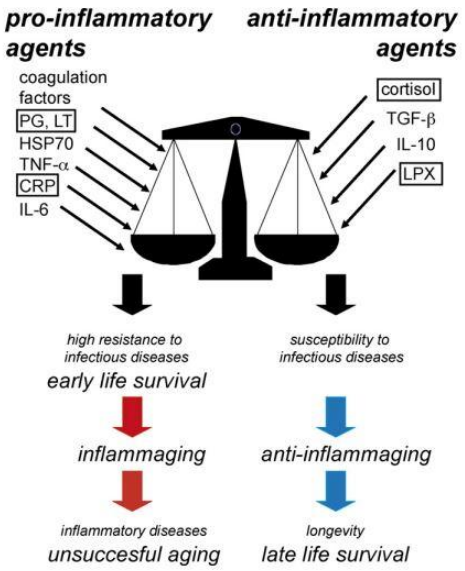


Fig. 9 The balancing between pro- and anti-inflammatory agents. Efficient inflammatory responses can confer high resistance to infectious diseases, but also an increased susceptibility to inflammation-based diseases later in life. On the other side, low inflammatory responses, while rendering more susceptible to infectious diseases, can confer a survival advantage in old age. (Franceschi et al., 2007)

“Inflammaging" is determined by the antigenic load that acts on the body throughout life and it is based on the assumption that the immune response and the targeted one against stress are equivalent and that the antigens should be regarded as a particular category of stressors. (Franceschi et al., 2000)

Inflammaging plays a crucial role in human health and ageing since the majority of age-related diseases such as metabolic syndrome, cardiovascular diseases, hypertension, type 2 diabetes, Alzheimer etc. share an inflammatory pathogenesis (Santoro et al., 2013). Moreover several geriatric conditions such as osteoporosis (Lencel and Magne, 2011), sarcopenia (Pedersen et al., 2009), or frailty (De Martinis et al., 2006) are highly influenced by inflammation.

Inflammation is not only a local, tissue restricted event but could also be a systemic process involving a variety of tissues (adipose tissue, muscle), organs (brain, liver), systems (immune system) and ecosystems (gut microbiota) of the body (Cevenini et al., 2010; Cevenini et al., 2013, Santoro et al., 2013) being a crucial component of immunosenescence.

In recent years is emerging more and more the role of N-glycomic profile in the blood and its age related changes associated with inflammaging as it will be described.

A N-glycan (N-linked oligosaccharide) is a sugar chain covalently linked to an sparagine residue of a polypeptide chain. The linkage commonly involves a GlcNAc residue and the consensus peptide sequence Asn-X-Ser/Thr. Glycans are involved in a wide range of biological processes due of their mass, shape, charge and other physical properties. Glycan chain structures are not encoded directly in the genome but are secondary gene products unlike protein sequences (Vanhooren et al., 2010)

On the basis of which mechanisms N-glycans are linked to inflammation? The alteration of glycosylation reactions can lead to agalactosylated N-glycans formation of decorating immunoglobulins G (IgG) and other glycoproteins (Vanhooren et al., 2010; Santoro et al., 2013). These IgGs are also called IgG-G0 and seem to be associated to several pathological conditions such as rheumatoid arthritis and other autoimmune diseases along with cancer, in which inflammation has a leading role (Dall’Olio et al., 2013, Santoro et al., 2013). According to Dall’Olio et al., proposed model of correlation between IgG-G0 and

pathogenesis of inflammatory conditions and aging (Fig.10) it is thought that the systemic inflammatory condition may alters the glycosylation machinery of antibody-producing cells, leading to increased expression of agalactosylated and poorly sialylated IgG. The abnormally glycosylated IgG activate different effector branches of the immune system causing the amplification of inflammatory signals.

The mechanism underpinning IgG-G0 and inflammation link is not clear.

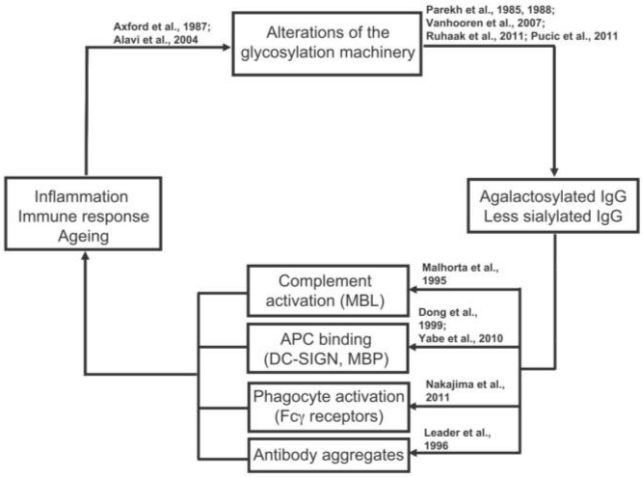


Fig. 10 Proposed model of IgG-G0 in the pathogenesis of inflammatory conditions and aging (Dall'Olio et al., 2013).

1.2 MARK-AGE PROJECT

1.2.1 MARK-AGE Project background

Many evidences indicate that the rate of ageing differs significantly between members of the same animal species, including humans. In other words, the "biological age" may differ from the "chronological age". The "demographic explosion" of which we are witnesses requires urgent solutions. It is in this perspective that ageing biomarkers fit. But what does is mean with the term "ageing biomarker"? An ageing biomarker essentially is an age-related change(s) in body function or composition that could be used as measure of "biological" age and the ageing rate.

The purposes for which these biomarkers could be used are very numerous:

1. Early detection of subject at high risk of developing age –related pathologies

2. Assessment of the efficacy of pharmacological, dietary or physical interventions.

The European Project MARK-AGE is not the first study that deals with this subject and during the years several exponent of scientific world tried to identify, without success, useful biomarker able to determine the "biological age". The reasons behind these failures are attributable to:

1. No Europe-wide study has been conducted so far so weak statistical power.

2. Old or very old probands were recruited and this made difficult to differentiate between real biomarkers of ageing from biomarkers of incipient or overt disease. Furthermore using exclusively cohorts of old volunteers the obtained data weren't informative for middle aged subject

3. Studies focused on single candidate biomarkers etc.

1.2.3 MARK-AGE Consortium

The MARK-AGE consortium consists of 26 Beneficiaries coming from 14 European countries (**Austria, Belgium, Danmark, Finland, France, Germany, Greece, Italy, Netherland, Poland, Romania, Spain, Switzerland e Great Britain)**, comprising **21 non-profit organizations** (universities and public research institutes), **3 small and medium sized enterprises** (SMEs), and **2 large companies** (Table 1). The project is coordinated by Prof Alexander Bürkle, University of Konstanz, Germany. The Scientific and Project manager is Dr. Maria Moreno Villanueva, University of Konstanz, Germany.

Beneficiary Number	Beneficiary name	Beneficiary short name	Country
1 (co-ordinator)	Universität Konstanz	UKON	Germany
2	BioTeSys GmbH	BioTeSys	Germany
3	Fundación Centro Nacional de Investigaciones Oncológicas Carlos III	CNIO	Spain
4	DNage B.V.	DNage	The Netherlands
5	Erasmus Universitair Medisch Centrum Rotterdam	Erasmus MC	The Netherlands
6	Facultés Universitaires Notre-Dame de la Paix de Namur	FUNDP	Belgium
7	Translational Medicine Cranfield Health	Cranfield	UK
8	Oesterreichische Akademie der Wissenschaften	OEAW	Austria
9	Istituto Nazionale Riposo e Cura per Anziani	INRCA	Italy
10	Nestec SA	NESTEC	Switzerland
11	National Hellenic Research Foundation	NHRF	Greece
12	Instytut Biologii Doswiadczalnej im. M. Nenckiego PAN	NENCKI	Poland
13	Institut National de Gerontologie si Geriatrie Ana Aslan	NIGG	Romania
14	Rijksinstituut voor Volksgezondheid en Milieu	RIVM	The Netherlands
15	StratiCELL Screening Technologies SA/NV	StratiCELL	Belgium
16	Aarhus Universitet	AU	Denmark
17	Aston University	UASTON	UK
18	Vlaams Instituut voor Biotechnolgie vzw	VIB	Belgium
19	Universität Hohenheim	UHOH	Germany
20	Martin-Luther Universität Halle-Wittenberg	MLU Halle	Germany
21	Alma Mater Studiorum – Università di Bologna	UNIBO	Italy
22	Unilever UK Central Resources Limited	UNILEVER	UK
23	Università degli Studi di Roma „La Sapienza“	UNIROMA	Italy
24	Université Pierre et Marie Curie – Paris 6	UPMC	France
25	Academisch Ziekenhuis Leiden – Leids Universitair Medisch Centrum	LUMC	The Netherlands
26	Tampereen Yliopisto	UTA	Finland

Table 1 MARK-AGE Beneficiaries

1.2.3 MARK-AGE strategy and major goals

The major goals of the MARK-AGE Project are the following:

1. **To perform a comprehensive Europe-wide population-based study** enrolling about 3,700 volunteers aged 35-74 years and representative of several different European geographical region with the purpose of identify human ageing biomarker.

2. **To recruit three large groups of subjects** from different European countries

a. **2320 R**andomly recruited **A**ge-**S**tratified **I**ndividuals from the **G**eneral population **(RASIG)** age range 35-74 years, belonging to both sexes Equal numbers of men and women will be enrolled, comprising in the following age classes: 35-39 yrs, 40-44 yrs, 45-49 yrs, 50-54 yrs, 55-59 yrs, 60-64 yrs, 65-69 yrs, 70-74 yrs. The volunteers are recruited as representative of the "normal" or physiological aging.

b. **700** Offspring of long-living parent belonging to a family with long living sibling(s) already recruited in the framework of the GEHA project ("GEHA offspring" or simply GO) recruited together with their spouses as controls ("GEHA Offspring Spouse" or simply SGO) aged 55-74 years. These subjects are recruited as representative of "successful" or decelerate aging.

c. A small number of patients with **Progeroid Syndromes (**Cockayne, Werner and Down Syndromes), characterised by accelerated "segmental" ageing.

3. **To extract a robust set of biomarkers of human ageing** and to derive a model for healthy ageing and also able to predict "bioage".

4. **To follow up a limited random sample of probands in a small longitudinal study**, compatible with the time constraint of the project (5 years) by re-sampling 12% of the recruited subjects (RASIG, GO, SGO) (about 450 subjects) and 50% of DS after 4 years.
5. **To collect anthropometric, demographic, clinical and social data on probands** and to obtain fasting samples of blood, buccal mucosal cells and urine.

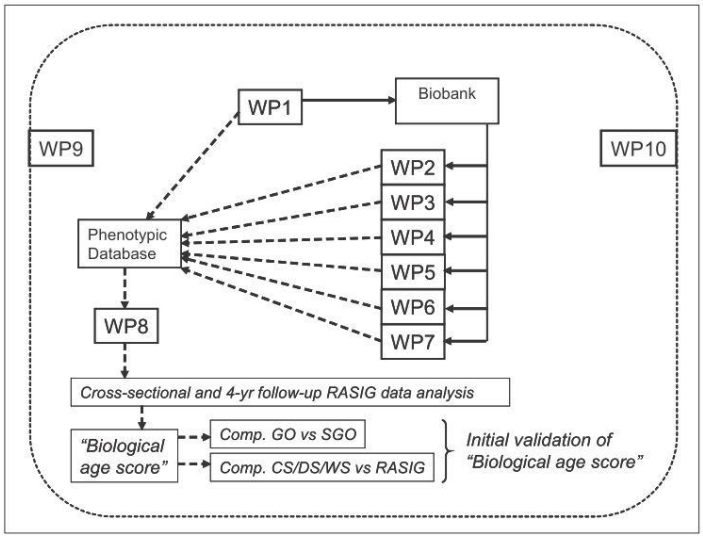


Fig. 11 MARK-AGE overall strategy of the work plan

The candidate biomarkers included in the analysis are (Table 2):

1. **"classical"** ones validated by several smaller studies
2. **"new/specialised"** ones, based on preliminary data obtained in small-scale studies
3. **"novel"** ones, based on recent research on mechanistic aspects of ageing, conducted by project beneficiaries.

Table 2. MARK-AGE Candidate biomarkers

Classical physiological parameters

Body mass index
Waist and hip circumference
Blood pressure at rest
Heart rate at rest
Lung capacity- FEV1
Lung capacity- FVC (forced expiratory vital capacity)
Near vision
Physiological postural equilibrium (maintenance of standing, tandem and semi-tandem position)
Five -times chair standing
Handgrip strength

Clinical chemistry analyses:

Blood urea nitrogen and creatinine
Fasting glucose and fasting insulin
Glycosylated hemoglobin (A1C)
Albumin and serum protein concentration
Fasting triglycerides and free fatty acids
Total cholesterol, HDL and LDL-cholesterol
Noradrenalin, serotonin and 17-hydroxycorticosteroid
C-reactive protein (CRP) and fibrinogen
Serum amyloid A and P, and pentraxin 3
Adiponectin
Testosterone (will be measured in males only)
Prostate specific antigen (PSA)

Specialised tests established by Partners

Cellular poly(ADP-ribosyl)ation capacity and DNA repair in PBMC
DNA methylation status in PBMC
Telomere length in PBMC
Changes in mitochondrial DNA in blood cells
APOE genotype
Vitamin levels in mucosal cells
Zn, Cu, Se and Fe in plasma and PBMC; metallothionein expression
Oxidative stress markers
Glycation
Serum glycans
Cytokines
ApoJ/clusterin
Proteasome, methionine sulfoxide reductases
Immunity against specific pathogens
Autoantibodies
Damage-Induced Cell Death and Activation-induced cell death in lymphocytes
Thymic output
Serum lipids including LDL particle size

Novel biomarkers yet to be established

Serum and PBMC proteomics
Metabonomics
Gene expression and protein profiling of blood cells (B cells, T cells) exposed to physiological oxygen tension
Proteins secreted by endothelial cells and fibroblasts
Biomarkers of ageing in the mouse and in Cockayne's syndrome patients
microRNAs

Since not all biomarkers are of equal weight only the best ones were matched and used for measuring the biological age.

1.2.4 Training

The scientific success of this Large-Scale Integrating Project, comprising 26 Beneficiaries, depends on the internal data coherence, integration of the work to be performed by each participant. Training activities have been based mainly on two main programmes:

(1) Training through research

(2) Specialized courses at summer schools.

During the first and the fourth project year, one-week summer schools for postdocs and PhD students involved in the project have been held.

The first summer school was held jointly with the FP6 Concerted Action LINKAGE on 29 June - 03 July 2009 in Fréjus, France. The topics covered in this summer school was the following: model systems in research on ageing, molecular mechanisms of ageing, in vitro systems, ROS and ageing, DNA damage and repair, cancer and ageing, methodology for gene expression analysis.

The second summer school was held jointly with the conference series Neurobiology and Neuroendocrinology of Aging, on 29 July - 02 August 2012, Bregenz, Austria.

Training was also implemented by exchange of researchers, especially young researchers, between Consortium members, with an emphasis on the technology platforms. Within the consortium there are excellent opportunities for training in specialised techniques.

A dedicated training was organized with all the recruiting Beneficiaries for the teams involved in the recruitment, data collection and data entry. A thorough explanation and a practical demonstration of all the items included in the informed consent and case-sheet has been performed in order to standardize the procedures and minimizing the risk of operator errors.

2. AIM OF THE STUDY

This dissertation is part of the Integrated European Project "MARK-AGE – European Study to Establish Biomarkers of Human Ageing" (Seventh Framework Programme Theme Health-2007-2.2.2-3 Biomarkers of Ageing), whose aim is to identify a robust set of biomarkers of human ageing able to discriminate between chronological and biological ageing and to derive a model for healthy ageing and also able to predict "bioage".

The specific aims of this thesis are the following:

1. To illustrate **the recruitment of three selected groups of subjects** from different European countries

    a. **R**andomly recruited **A**ge-**S**tratified **I**ndividuals from the **G**eneral population **(RASIG)** age range 35-74 years, belonging to both sexes The volunteers are recruited as representative of the "normal" or physiological aging.
    b. Offspring of long-living parent belonging to a family with long living sibling(s) already recruited in the framework of the GEHA project ("GEHA offspring" or simply GO) recruited together with their spouses as controls aged 55-74 years. These subjects are recruited as representative of "successful" or decelerate aging.
    c. A small number of patients with **Progeroid Syndromes (**Cockayne, Werner and Down Syndromes), characterised by accelerated "segmental" ageing.

    The recruitment of Progeroid Syndrome patients will be only briefly mentioned since these individuals won't be considered for the analysis performed in this dissertation.

2. To perform **an accurate analysis of the health status** of elderly recruited GO-SGO and RASIG volunteers aged 60-79 years, verifying any possible dissimilarity in their aging trajectories.

3. To identify **a set of robust ageing biomarkers**.

4. To investigate **possible correlations between ageing biomarkers and health status** of recruited volunteer.

3. MATERIALS AND METHODS

3.1 PRELIMINARY ACTIVITIES TO THE RECRUITMENT

3.1.1 Selection of the Study Population

Three populations were recruited as follows (Capri et al. 2014):

1. **Subjects representing the "Normal" or "Physiological" aging: R**andomly recruited **A**ge-**S**tratified **I**ndividuals from the **G**eneral population or RASIG, covering the age range 35-74 years (both sexes).

2. **Subjects representing the "successful" or "decelerate" aging:** subjects belonging to **long living families, enriched of pro-longevity characteristics.** Offspring of long living siblings previously recruited in the framework of the GEHA "Genetic of Healthy Ageing" project (Skytthe et al., 2011). GEHA offspring" or **GO** were recruited together with their spouses (**SGO**) as best control to evaluate possible life style effects, since they shared the same environmental factors for many years with their partners. Age range: 55-74 years (both sexes).

3. **Subjects representing "accelerated" aging:** patients affected by **progeroid syndromes** (Cockayne, Werner and Down syndromes), characterised by accelerated "segmental" ageing.

Each recruitment unit focused their activity on different populations:

RASIG population: BioTeSys GmbH (Germany), University of Namur (FUNDP, Belgium), Österreichische Akademie der Wissenschaften (OEAW , Austria), Institute of Experimental Biology (NENCKI, Poland), National Hellenic Research Foundation centre (NHRF, Greece), University of Tampere (UTA, Finland) and University of Bologna ALMA MATER STUDIORUM (UNIBO, Italy).
GO/SGO population: University of Leiden (LUMC, Netherland), University of Tampere (UTA, Finland), NENCKI, UNIBO, FUNDP and NHRF.

Down Syndrome population: University of Bologna ALMA MATER STUDIORUM (UNIBO, Italy).

Cockayne and Werner Syndrome DNage B.V. (Netherland) and University of Konstanz (UKON, Germany) were in charge for the collection of existing blood samples from patients recruited by external collaborators

3.1.2 Inclusion and exclusion criteria

Inclusion criteria

1. **RASIG:** Randomly recruited age-stratified individuals from the general population (both sexes) aged 35-74 years, able to give informed consent and living in country of residence for longer than 50% of lifetime.

2. **GO and SGO:** Offspring (sons or daughters) of GEHA siblings and GO spouses (SGO) aged 55-74 years and able to give informed consent.

Exclusion Criteria

1. **Self-reported seropositivity for HIV, HBV** (except seropositivity by vaccination) **and HCV** (HBV and HCV seropositivity was assessed after blood collection).
2. **Presence of cancer and current use of anti-cancer drugs or glucocorticoids**.
3. **Less than 50% of lifetime spent in country of residence.**
4. **Inability to give informed consent**

3.1.3 Standardization of Recruitment Tools

All the beneficiaries involved in the recruitment had to prepare a set of standardized documents that includes:

a) **A standardized invitation letter** that was sent to all the eligible volunteers (RASIG, GO and SGO) as first approach.

b) An **Informative Sheet** containing exhaustive information, written in a clear and understandable way, with the purpose of explaining, the background, goals and the study procedures (interview, physical examination and sampling of blood, urine, buccal mucosa cells and saliva).
c) **A Synopsis** summarizing the focal points of the project.
d) **An Informed Consent form** redacted in compliance with the local legislation in the different European countries involved in the recruitment.
e) **Four Questionnaires,** that have to be uniformly used for all the recruited subjects

3.1.4 Phenotipic questionnaires design

Four standardized questionnaires were produced using part of the questions which were consolidated in previous European studies on elderly subjects and younger controls (such as GEHA).

Interview Questionnaire Part I-A (to be filled out by all study participants at home) containing questions regarding:

1. **Basic biographic information**: family composition, parents' causes of death, marital status, education level, main occupation (classified according to ISCO classification), living condition and social network.
2. **Life style**: smoke, food habits and alcohol intake.
3. **Basic Health Information:** SF-36v1 Health Survey, present and past pathologies, use of prescribed medicines, falls, hospitalization and change of weight in the last 12 month, vaccination against tetanus and HBV, use of hormonal contraceptives or hormone replacement therapy in the last year (females only), presence of presbyopia.

Interview Questionnaire Part I-B (to be filled out only by over 60 years participants at home) including:

1. **Katz's ADL** (Activity of Daily Living) (Katz et al., 1970).
2. **Questions from the Nagi-scheme** (Nagi SZ, 1976) regarding ability of reading newspaper without glasses or other aids, clearly recognize someone 4 metres away without glasses, hearing ability without aids, half a kilometer/a quarter of a mile

walking without aids, going up and down a flight of stairs without anyone's help, doing any kind of light housework or exercise, going outside with or without anyone's help.)

Interview Questionnaire Part II-A (to be filled out by the interviewer) consisting in:

1. **Blood pressure measurements** (at the beginning of the interview and after the cognitive assessment)
2. **Cognitive and mood evaluation:**
    a. **15 Picture Learning Test (15-PLT):** it assessed the immediate and delayed memory function. 15 pictures of well-known items were shown to the participants and then asked to recall as many as possible. The test consists in three consecutive and repeated trials and a postponed recall after 20 minutes. The total number of correct answers after three trials was defined as the immediate recall while the number of correct answers after 20 minutes as delayed recall. Outcome parameters were the number of correct pictures remembered immediately and after the pause. The number of incorrect pictures for each trial was reported. A low score indicates worse cognitive performance. (Brand and Jolles, 1985).
    b. **Stroop-Colour-Word-Test**: it was used to test selective attention. The test consists in three sheets showing 40 stimuli each, which the subject was asked to read or name as quickly as possible: (1) colour names, (2) coloured patches, and (3) colour names printed in incongruously coloured ink. Difficulty of part 3 is mostly due to the need to discard irrelevant information (verbal), in favour of a less obvious aspect (colour naming). This phenomenon is also known as cognitive interference. The main outcome was the times needed for each of the three trials and a higher score denotes a worse performance (Stroop, 1935).
    c. **Digit-Symbol Substitution Task (DSST)** is a psychometric test and it was used to evaluate processing speed and sustained attention. In this test digits were presented and the participants were asked to write down the corresponding symbols in a blank space according to a provided key. Outcome parameter was the number of correct written symbols within 90 seconds. A low score denotes worse cognitive performance (Lezak et al., 2004).
    d. **Zung Self-Rating Depression Scale** is a short (20 items) self-administered scale able to assess the level of depression in patients diagnosed with

depressive disorder. It is widely used as a screening tool, covering affective, psychological and somatic symptoms associated with depression. It is composed by ten positively worded and ten negatively worded questions. Each question is scored on a scale of 1 through 4. A higher score indicates a more depressed status (Zung, 1965).

Interview Questionnaire Part II-B (to be filled out by the interviewer only with over 60 years participants) consists in:

- **Mini-Mental State Examination – SMMSE** (Molloy et al., 1991) is the most commonly used instrument for screening cognitive function. The test is not suitable for making a diagnosis but can be used to indicate the presence of cognitive impairment.

3.1.5 Local Ethics Committee authorization

One of the main goals during the first year of the project was to obtain the approval of the local ethical committees. For this reason a standardized version of the necessary documents (invitation letter, informative sheet, informed consent, questionnaires etc.) was prepared. Due to different ethical requirements between countries, it was necessary to make appropriate adjustments in order to be compliant with the different legislations. All the documents were then translated into the national language and submitted to local Ethical Committee for approval.

3.1.6 The TRY-Phase

In order to evaluate that all procedures were applied in the correct way and to monitor the '"time effect" between the sample collection and the beginning of the processing, a test phase called “TRY-Phase” was performed. The entire standard operating procedure, from recruitment (informed consent, questionnaires), sample collections and processing, subject coding to entry data and analytical measurements was simulated. For this purpose, a group of volunteers who were excluded from the MARK-AGE study were selected.

3.2 RECRUITMENT STRATEGIES OVERVIEW

3.2.1. Recruiting Units different approaches

In population-based studies the set-up of volunteers' recruitment strategy is a very crucial phase perhaps the most challenging part of a clinical research on which depend the success of the experimentation since problems with recruitment can disrupt the timetable for a research project (Patel et al. 2003).

The difficulty of this process is further increased when recruitment needs to be planned in different nations as it has to take account of geographical, social and cultural differences between the countries involved. For this reasons it was decided that each recruiting unit would pursue different approaches, in order to reach the largest number of potential volunteers. Consequently specific and country oriented strategies were applied according to the population to recruit (GO, SGO or RASIG volunteers).

Every unit previously involved in GEHA Project recruitment was able to take advantage of past references in order to directly locate possible candidates or at least tried to reconnect with families in order to reconstruct the network of contacts useful for recruitment.

Though each partner adopted different strategies the use of mass media was chosen as preferential way of communication and dissemination. In fact the involvement of newspapers, radios and local TVs along with production of advertising material (flyers, posters etc.) played a decisive role in the success of MARK-AGE recruitment.

In the table below are summarized the main strategies adopted by each recruitment centres (Table. 3)

Beneficiary	Population	Dissemination and Strategy of recruitment
BioTeSys GmbH (GERMANY)	RASIG	Newspaper articles, information evening at the town hall together with the governing mayor; registration office (letter /flyer), volunteers known from other trials conducted at BioTeSys, a little word-of-mouth recommendation.
FUNDP/ StratiCELL (BELGIUM)	RASIG/GO/SGO	Contacted an open-university for persons belonging to the 3rd age and all societal horizons; the Services of the Human Resources of the City of Namur, the Univ. of Namur, Univ. Clinics of Mont-Godinne, and Regional Hospital Centre, dealing with all sorts of personnel; organised press conference at Univ. of Namur (many press articles, interviews on national radios, local TV news, national TV programme on ageing). GEHA references for GO list
LUMC (NETHERLANDS)	GO/SGO	Drafted a list with a number of picked nominatives (GEHA references) .
NHRF (GREECE)	RASIG/GO/SGO	Contact by email all the personnel of Research Institutes of Athens. Contact by phone call all the GEHA siblings giving information to GO/SGO on MARK-AGE, sending them the informative sheet by post or fax. Contact by phone call all our personal acquaintances giving information on MARK-AGE.
NENCKI (POLAND)	RASIG/GO/SGO	Obtained addresses of 3200 RASIG from Ministry of Interior and Administration according to PESEL (National Electronic Census Number System); sent 240 letters of invitation , 22% responders feedback by phone or e-mail GEHA references for GO list
UIBK (AUSTRIA)	RASIG	Articles in a very common Tyrolean daily newspaper; dissemination on local TV news and articles in other newspapers and magazines.
UNIBO (ITALY)	RASIG/GO/SGO/ DS	GO and SGO were recruited before RASIG: drafted a list with a number of picked nominatives GEHA references for GO list. RASIG: population of PIANORO (a small city of about 17,000 inhabitants near Bologna); contacts with the Mayor and the District of Public Health. The project was illustrated to the Pianoro population including the local general practitioners; and disseminated to the local newspaper and TV. Flyers were also prepared and distributed in the local supermarkets. Another group of volunteers was recruited by an advertisement on the website of the University of Bologna. DS and their family were contacted by specific associations in Bologna
UTA (FINLAND)	RASIG/GO/SGO	Newspapers, local TV announcements. GEHA references for GO list

Table 3 Mark-Age different recruitment strategies among the beneficiaries (Capri et al. 2014)

3.2.2 The UNIBO experience

The UNIBO experience was quite peculiar since it was in charge of recruitment of GO, SGO, RASIG volunteers' along with subjects affected by Down Syndrome and this required the development of accurate and targeted approaches.

In UNIBO, MARK-AGE recruitment started with the identification of possible eligible GO and SGO candidates. This research was carried out using a list with a number of picked nominatives drafted during GEHA recruitment. In order to optimize the search of participants, avoiding energy dispersal and waste of valuable time, an *ad hoc* recruitment procedure was developed by recruiters (Fig. 12).

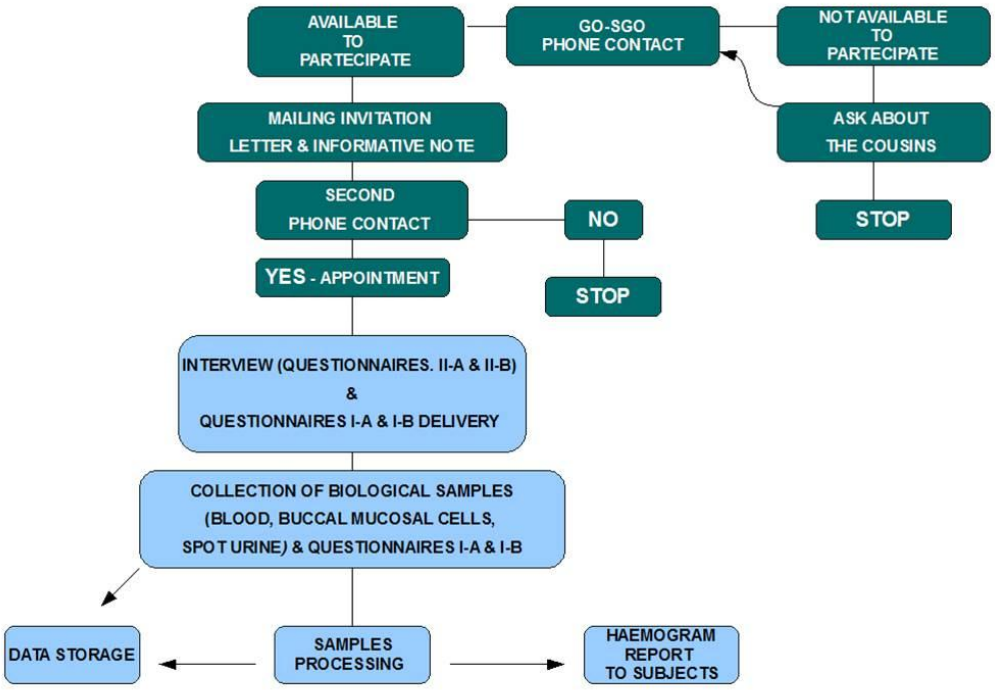


Fig. 12 GO-SGO recruitment procedure

Every possible candidate was firstly contacted by phone, sometimes using their old GEHA parents as intermediaries. If the subject refused to participate, information about the presence of cousins that could be available were asked otherwise in case of acceptance the prepared invitation letter and the informative note were sent by regular mail or email. After some days the possible candidate were contacted again by phone. If the response was positive an appointment was scheduled otherwise the search started again.

RASIG recruitment was mainly carried out in Pianoro, a little town near Bologna and partially between Bologna University employees. In order to obtain a successful recruitment of RASIG subjects in Pianoro the elaboration of communication strategies was necessary. The involvement of University of Bologna, Mayor and City Council of Pianoro, Public Health District, General Practitioners (GPs), voluntary associations was indispensable to establish medical-scientific synergies and to set-up a connections network where citizens could find information on MARK-AGE project. The dissemination was accelerated with the creation of informative material (flyers and posters) left near institutional centers and aggregation places such as City Council, local Health Centre, General Practitioners ambulatories, pharmacies, supermarkets etc.

The recruitment between University of Bologna employees or their relatives was promoted and facilitated by an advertisement on the official University website.

Also with RASIG volunteers an *ad hoc* procedure was developed (Fig. 13)

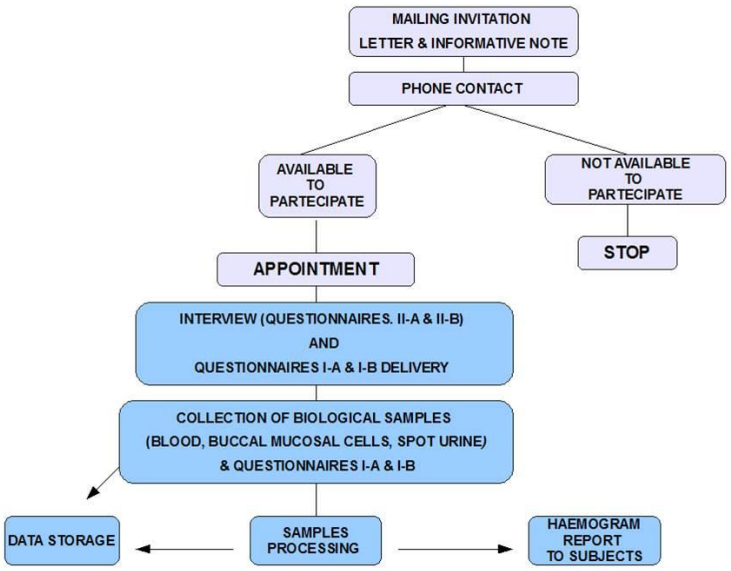


Fig. 13 RASIG recruitment procedure

UNIBO team was the unique centre in charge of recruitment Down Syndrome patients and this required the development of accurate and targeted approach. The subjects affected by Down Syndrome and their family were contacted by specific associations in Bologna.

3.3 QUESTIONNAIRES ADMINISTRATION AND SAMPLES COLLECTION PROCEDURES

The questionnaires administration and samples collection was preferably performed at University of Bologna whenever was possible or at volunteer's home. Before collecting any kind of information, the informed consent was firstly explained in every detail to the participant and then signed by candidate and by the recruiter (a MD or a nurse or specifically trained biologist). From every participant were collected 50 ml of blood by phlebotomy, after overnight fasting (water and medications excluded), urine (at least 19 ml) and buccal mucosa cells. From the total amount of blood collected, 2.7 ml of whole blood was sent to the local clinical chemistry laboratory for blood counts. After the blood withdrawal and breakfast the questionnaires were administered and questionnaires completed at home were thoroughly checked.

3.4 BIOLOGICAL SAMPLES PROTOCOL

3.4.1. Coding protocol

All the collected data, personal information contained in the questionnaires or obtained from biological samples are sensible data and as such they require confidentiality that has to be ensured by complying with the legislation. All the samples and the questionnaires were coded at each recruitment centre by assigning to each volunteer a unique number consisting in 7 digits based on coding scheme (the first two numbers defining the MARK-AGE recruiter centre followed by a five digit number), called PrimaryID (PID). The code was held exclusively at the recruitment centre in compliance with national legislation. All data obtained from the laboratory analysis were entered into the bio-bank and the central database only using a secondary code (PID2) generated and held by Beneficiary 1 only and not released to anybody. Only PID2-coded biological samples were exchanged between Beneficiaries for *ad hoc* analysis.

3.4.2. Sample processing protocol

In order to standardize the sample collection all the recruitment centres were supplied with

pre-labelled material directly by the Biobank (Moreno-Villanueva et al. 2014).

1. **8 blood monovettes** (ranging from 2.7 to 9 ml) containing lithium heparin, EDTA or clotting activator.

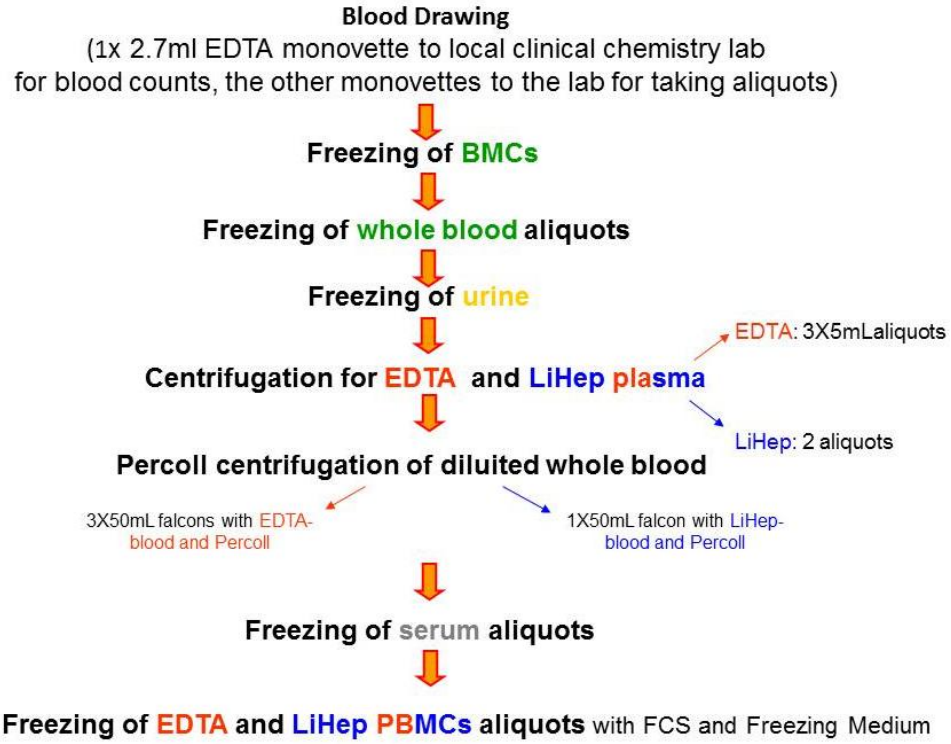
2. **29 cryo tubes** (ranging from 0.5 to 15ml)

3. **1 kit** devoted to buccal mucosa cells collection and consisting of a special toothbrush, a stabilizer solution and empty tubes.

Awaiting the processing:

- The monovettes were stored at room temperature in the dark (before starting of the preparation protocol).
- Urine was stored at 4°C or on ice before freezing
- Buccal Mucosa Cells suspension was stored at 4°C or ice cooling, in the dark for no longer than 10 hours before freezing

The processing protocol was performed as follows:



For each volunteer the time necessary to complete the whole procedure was about 2-2.5 hours.

3.4.3. The Biobank

The MARK-AGE Biobank represented a focal point of all project since its activity was fundamental for several aspect of MARK-AGE processes. It was located at the University of Hohenheim, Germany and precisely at the Institute of Biological Chemistry and Nutrition. The main purpose of MARK-AGE Biobank was to provide standardized equipment for samples collection to all the recruitment centres, to manage samples incoming and outcoming (from the recruitment centers and to the analytic partners), sample re-labelling and splitting and storage along with sample tracking (Moreno-Villanueva et al. 2014).

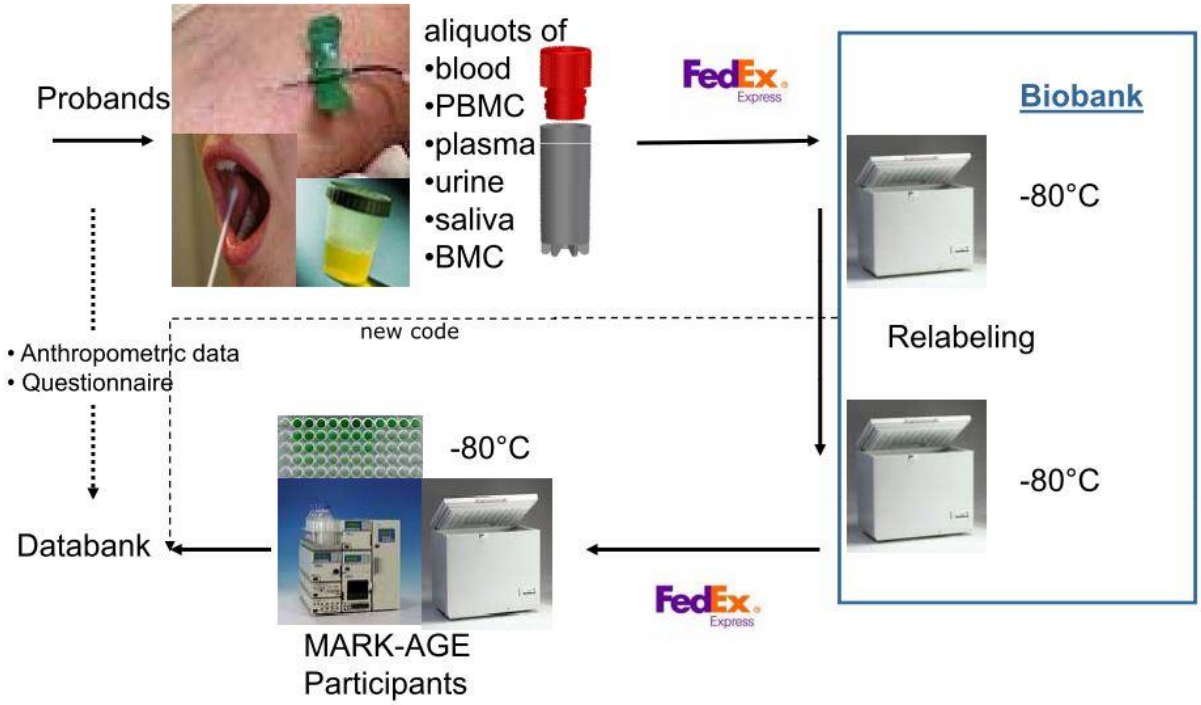


Fig. 14 Role of Mark-AGE Biobank

3.5 MARK-AGE DATABASE

All the information collected with questionnaires, anthropometric data along with biological data obtained by blood, urine and buccal mucosa cells belonging to each MARK-AGE volunteer were stored electronically in the MARK-AGE database (Fig. 15). All the data were put in through a secure internet link.

The entering data procedure was tested repeatedly in order to prevent any problems during the phase of active recruitment

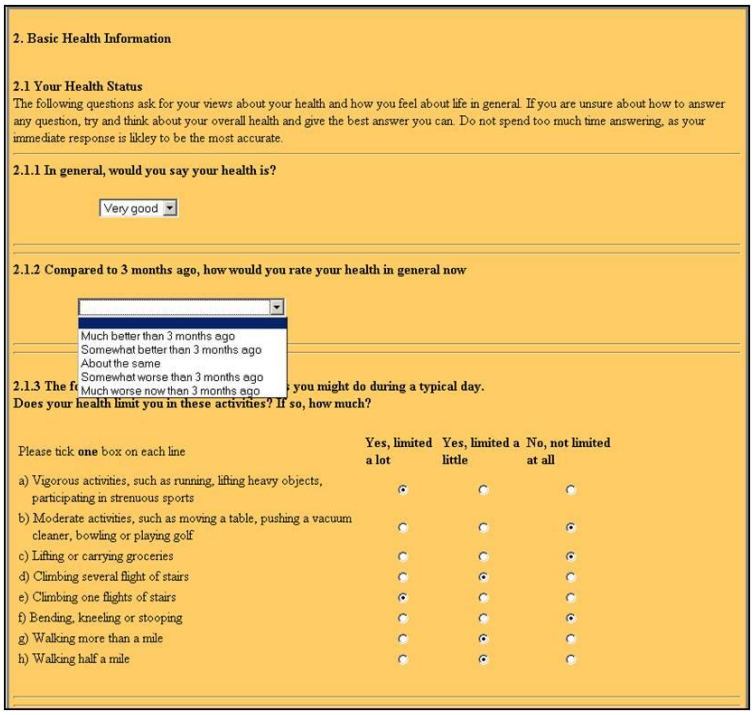


Fig. 15 Example of MARK-AGE database interface

3.6 PHENOTIPICAL VARIABLES ASSESSED BY MARK-AGE QUESTIONNAIRES AND INCLUDED IN THE ANALYSIS

3.6.1 Socio-demographic Factors

Data regarding present marital status, level of education and years of schooling, occupation, living condition (living at home or not) and cohabitation were included. Regarding the marital status the category "married" also took account of the live-in partners. The occupation was evaluated according to the ISCO classification (International Standard Classification of Occupation) and subjects were divided in "White Collars" (legislators, senior officials and

managers; professionals; technicians and associate professionals, clerks; service workers and shop and market sales workers; military), “Blue Collars ”(skilled agricultural and fishery workers; craft and related trades workers; plant and machine operators and assemblers; elementary occupations) and housekeepers (Cevenini et al., 2013).

3.6.2 Lifestyle Factors

Consumption of alcohol and smoke were considered. Regarding alcohol only everyday use was indicated. Concerning smoke the participants were divided in never smokers, former smokers, and smokers.

3.6.3 Health Status

Present health status was assessed asking subjects by which of the following diseases were affected:

- Vision impairment
- Hearing impairment
- Neurological diseases
- Angina pectoris
- Irregular heart beat
- Heart failure
- High blood pressure (hypertension treated with prescribed drugs)
- Venous insufficiency in legs/leg ulcers
- Pain in leg during walking
- Chronic respiratory diseases (COPD, asthma, chronic bronchitis etc.)
- Chronic renal failure
- High cholesterol
- Diabetes
- Hyperthyroidism
- Hypothyroidism
- Autoimmune Diseases (SLE, rheumatoid arthritis etc.)
- Cirrhosis of the liver
- Osteoporosis

- Arthritis
- Serious memory impairments
- Other mental health problems

Other health problems not mentioned in the previous list were also recorded. The presence of past pathologies such as pneumonia, myocardial infarction, stroke, cerebral thrombosis/hemorrhage, malignant tumor and hip fracture was registered. The use of prescribed medicines along with the hospitalization (with an overnight stay) and the number of falls occurred in the last year were considered. Moreover the number of pathologies together with the number of medications used was analyzed.
Self-reported health was classified using the following question from SF-36v1 Health Survey: *"In general, would you say your health is?"* with five-choice response scale (excellent, very good, good, fair and poor). Furthermore the subjects were clustered in "Healthy" (those who answered excellent, very good and good) and "Not Healthy (those who answered fair and poor).

3.6.4 Disability

The assessment was performed only on subjects over 60 years old using the Katz Index of activities of daily living (ADL) six-items scale (Katz et al., 1970). Only bathing, dressing, toileting, transfer and feeding were considered for categorization according to literature (Fillenbaum GG, 1996). Continence was analyzed separately. In compliance with Katz definitions (Katz et al., 1970) obtained data were distributed on a three level scale:

Severely disabled (ADL = 0-2)
Moderately disabled (ADL = 3-4)
Not disabled (ADL = 5)

The disability analysis was integrated with some questions from the Nagi-scheme (Nagi SZ, 1976): reading newspaper without glasses or other aids, clearly recognize someone 4 metres away without glasses, hearing ability without aids, half a kilometer/a quarter of a mile walking without aids, going up and down a flight of stairs without anyone's help, doing any kind of light housework or exercise, going outside with or without anyone's help.

3.6.5 Functional status

Physical performance was assessed with Chair Stand Test and Handgrip strength measurement.

For Chair Stand Test the subjects were divided in three categories: able to perform the test, stop after 1-4 times and not able at all. Only the participants able to complete the test were considered for the analysis. Results of five times chair stand were reported as number of seconds spent to complete 5 chair stands.

Handgrip strength was measured using a hand-held dynamometer (SMEDLYS' dynamometer, Scandidact, Kvistgaard, Denmark) for three performances with each hand. The best performance of the dominant hand, in the able to perform volunteers, was used for the analysis (Gueresi et al., 2013).

3.6.6 Anthropometric measurements

The height measurement was performed with a common metre while the weight was measured using a common balance (SECA Mod. 761). Body mass index was calculated on weight and height data (K/m^2). The subjects were divided for gender.

3.6.7 Cognitive Status

For the analysis were used only raw scores.

Mini-Mental State Examination (SMMSE)

This assessment (Molloy et al., 1991) was performed only on over 60 years old subjects The participants were divided in: 1."severely impaired" (0–17 points), 2. "mild impaired" (18–23 points) and "not impaired" (24–30 points) (Nybo et al., 2003).

15 Picture Learning Test (15-PLT)

The immediate and delayed memory function was assessed by the 15-Picture Learning Test

(Brand and Jolles, 1985). For the analysis were considered the sum of corrected pictures recalled immediately during the three consecutive trials and also the number of corrected pictures recalled after 20 minutes. A low score indicates worse cognitive performance. Only the tests reported as complete and reliable were used for the analysis.

Stroop Colour Word Test

The scores were the seconds necessary to complete the three trials of Stroop Colour Word Test (Stroop, 1935). A high score indicates worse performance.

Digit Symbol Substitution Task (DSST)

The processing speed assessed by the Digit-Symbol Substitution Task (Lezak et al., 2004) was scored using the number of correct digit-symbol combinations within 90 seconds. A low score indicates worse. Only the tests reported as complete and reliable were used for the analysis.

3.7 STATISTICAL ANALYSIS

The database was cleaned to remove all the subjects whose nominal age was not in the appropriate range of the project. Most analysis was performed on a subset of the subjects of age over 60, to balance the effect of age and between different groups.

For each test the subjects missing relevant parameter values were drop out.

All the linear regressions were performed with a multivariate robust linear regression with a Huber's T kernel and using age, gender and subject group as covariates.

The regressions plot were done aggregating data by time range and representing their mean and standard deviation to ease visualization, and the region of confidence of the regression tendency of age are represented as shades.

For categorical variable, the dependence with groups was tested with a Fisher exact test, found in the R statistical package. For categorical variable with dependence on continuous parameters like the age, a logistic test was performed, using age, gender and subject group as covariates.

Few variables, like methylation status of ELOVL and FHL2, were calculated as the composition of different, related, partial parameters that are known to be related to the same phenomenon and are strongly correlated. These variables were combined through a mean, and the resulting value was used for all the following analysis. This approach leads to more robust and significant results than the analysis with any of the single variables.

The analysis was performed with the Pyhton statistical libraries (statsmodels, seaborn, pandas, scipy) and the R statistical suite.

In the performed statistical analysis Bonferroni-Sidak correction for multiple comparisons was applied. For this reason **p values < 2.564e-04** will be considered statistically significant.

4. RESULTS

4.1 MARK-AGE RECRUITMENT ACCOMPLISHMENTS

4.1.1 MARK-AGE recruitment in all the European centres

As extensively explained before, the ambitious purpose of MARK-AGE project was to recruit a very consistent number of participants who were representative or assumed to have diverse rates of ageing in order to identify a group of ageing biomarkers able to quantify the biological ageing.

MARK-AGE goal was largely achieved as a total of 3387 subjects were recruited. In particular 2336 RASIG, 566 GO, 323 SGO, 53 Down-Syndrome, 50 Werner Syndrome and 59 Cockayne-Syndrome patients as shown in Table 4

BENEFICIARIES	RASIG		GO		SGO		DS+WS+CS
	Men	Women	Men	Women	Men	Women	
1 UKON (DE)							50 WS
2 BIOTESYS (DE)	172	189					
4 DNAGE (NL)							59 CS
6 FUNDP (BE)	122	138	36	44	17	23	
8 UIBK (AT)	200	201					
11 NHRF (GR)	202	211	8	16	4	3	
12 NENCKI (PL)	204	205	25	58	31	17	
21 UNIBO (IT)	201	200	55	53	26	37	53 DS
25 LUMC (NL)			47	71	64	43	
26 UTA (FI)	27	64	58	95	24	34	
Subtotal	1128	1208	229	337	166	157	162
			566		323		
TOTAL	2336		889				3387

Table 4 number of volunteers recruited by the different centers

RASIG population appeared to be well homogeneous distributed in the age classes and per gender (Fig16 and Fig18).

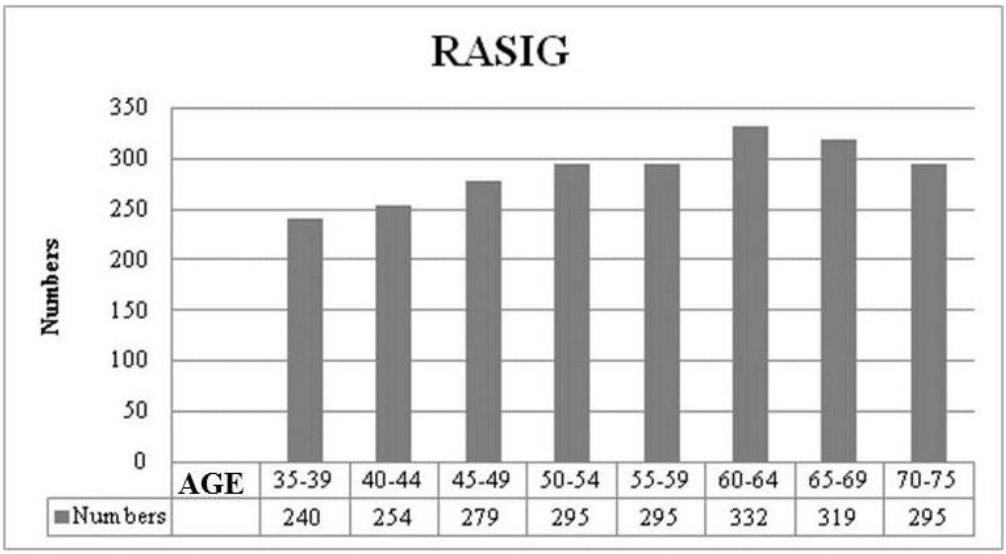


Fig. 16 RASIG distribution for age classes

Contrary to the initial expectations the number of SGO subjects was about half of GO and this was mostly due to the fact that several GO subjects was singles, divorced or widowed or simply SGO were not eligible or declined MARK-AGE invitation.

In spite of everything GO/SGO recruitment was a success since recruiters had to overcome many challenges in order to complete their task. As for RASIG participants also the distribution GO/SGO volunteers was homogeneous (Fig.17 and Fig.18).

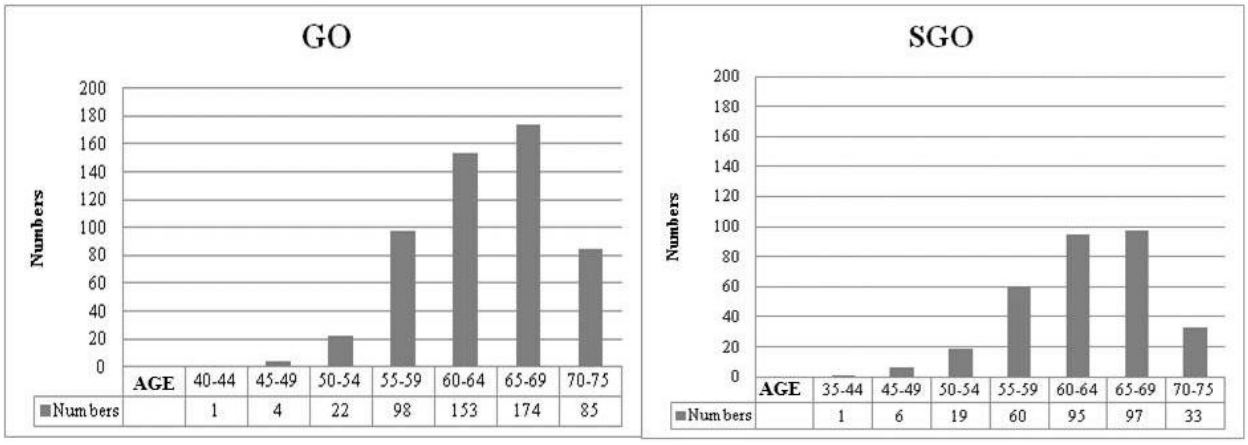


Fig. 17 GO-SGO distribution for age classes

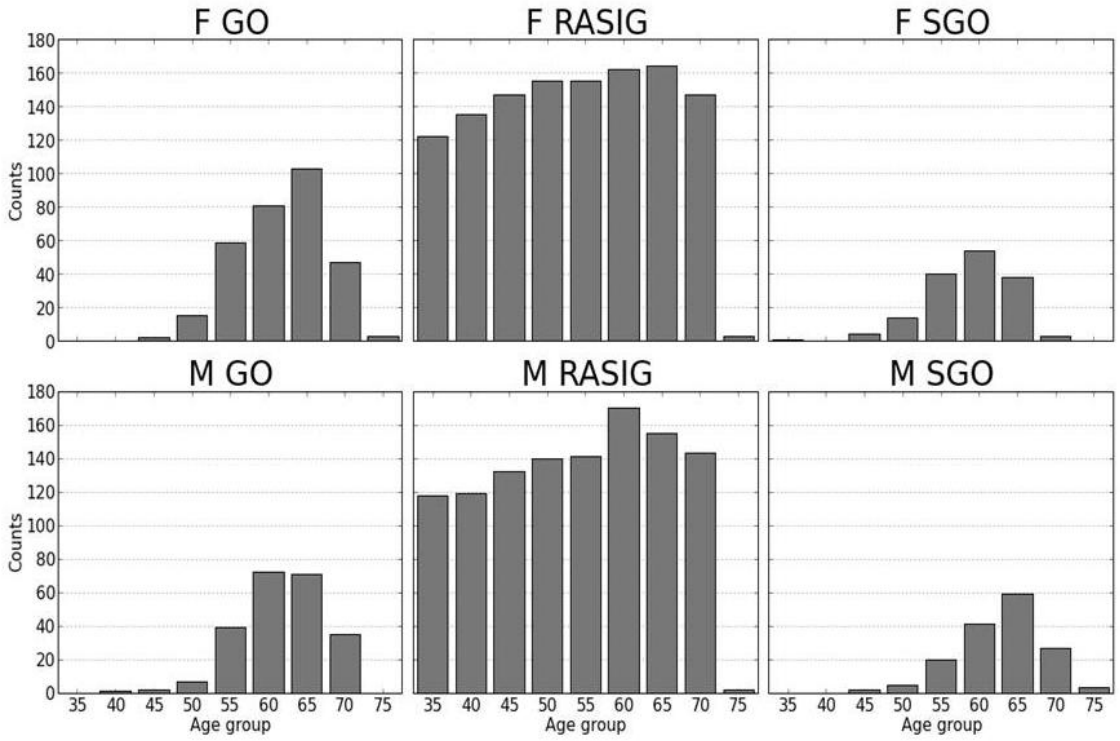


Fig. 18 Distribution of the sample for groups and gender

UNIBO recruitment unit was in charge of Down Syndrome individuals enrolment and was able to collect and characterize 53 subjects

Regarding the collection of existing blood samples belonging to Cockayne-Syndrome patients DNage B.V. and later on University of Konstantz (UKON, Germany) were responsible for the collection of about 59 samples.

4.1.2 MARK-AGE recruitment in UNIBO

The overall number of recruited volunteers in UNIBO was 627 (Table 5). It was undoubtedly an extraordinary result considering the complex procedures necessary to collect so different populations of GO, SGO, RASIG and Down Syndrome patients. In order to obtain the required number of volunteers a very large number of possible candidates was contacted and long distances were covered. Recruitment of GO e SGO subjects took place in almost the entire Emilia-Romagna region since it involved the counties of Bologna, Modena, Ferrara, Ravenna e Forlì-Cesena. The whole number of GEHA families contacted was 192 and 109 GO plus 64 SGO were enrolled. As happened in the other recruitment centers also in UNIBO

the search of SGO candidates was very difficult and this is the reason why they represented only the 58.7% of GO recruited in UNIBO.

RASIG recruitment was performed in the small town of Pianoro (a little city near Bologna) and within Bologna University employees and their relatives and friends. The total number of subjects contacted was 779, of those 482 amid Pianoro population while the other 296 were contacted among the Bologna University staff.

Volunteers affected from Down Syndrome were recruited involving specific associations, families along with psychologists and psychiatrists.

RECRUITMENT UNIT	RASIG		GO		SGO		DS
UNIBO	Men	Women	Men	Women	Men	Women	
	201	200	55	54	26	38	53
			109		64		
TOTAL	401		173				627

Table 5 Mark-Age recruitment in UNIBO

4.1.3 The Re-Sampling Phase

A re-sampling was performed on 108 volunteers from the whole study population, as shown in Table 6 and this was done to confirm the biological and analytical robustness of the measurements. University of Konstantz (UKON, Germany) randomly selected a list of PrimaryID of the recruited probands (GO, SGO and RASIG of both sexes) that had to be re-tested and sent it to all beneficiaries involved in the recruitment.

BENEFICIARIES	
	Subjects
1 UKON (DE)	
2 BIOTESYS (DE)	10
4 DNAGE (NL)	-
6 FUNDP (BE)	12
8 UIBK (AT)	17
11 NHRF (GR)	35
12 NENCKI (PL)	11
21 UNIBO (IT)	12
25 LUMC (NL)	11
26 UTA (FI)	-
Total	108

Table 6 number of subjects re-sampled

4.1.4 The Re-Testing Phase

After 3 years a mini-longitudinal study was performed re-testing a limited number of GO, SGO and RASIG subjects (only the 12% of recruited volunteers was involved) in order to validate the predictive power of specific biomarkers. 405 subjects in total were once again assessed using the complete standard procedure applied for the first assessment (Table 7).

BENEFICIARY	RASIG		GO		SGO		
	Men	Women	Men	Women	Men	Women	
1 UKON (DE)							
2 BIOTESYS (DE)	21	27					
4 DNAGE (NL)	-	-	-	-	-	-	
6 FUNDP (BE)	13	20	7	10	3	5	
8 UIBK (AT)	24	24					
11 NHRF (GR)	21	30	-	3	1	-	
12 NENCKI (PL)	28	41	1	2	-	-	
21 UNIBO (IT)			27	25	11	12	
25 LUMC (NL)			4	8	8	4	
26 UTA (FI)	1		9	10	4	3	
Subtotal	108	142	48	58	27	24	
Total	248		106		51		405

Table 7 Numbers of re-tested volunteers

Also in this case University of Konstantz (UKON, Germany) randomly selected the subjects for the re-testing.

4.2 CHARACTERIZATION OF THE SELECTED SAMPLE ANALYZED

4.2.1 Composition of the sample

The sample used in this dissertation was stratified on the basis of age ≥ 60 yrs, thus 1598 volunteers aged 60 – 79 years were constituted as follows: 1. **420 GO** (179 males and 241 females), 2. **231 SGO** (134 males and 97 females) and 3. **947 RASIG** (470 males and 477 females). The sample is homogeneously divided in males (49%) and female (51). The number of RASIG subjects divided per gender was similar, on the contrary GO and SGO were more unbalanced (Table 8).

Table 8 Composition of the analyzed sample (N=1598) divided for gropus, gender and age classes

	Total N	%
Gender		
Male	783	49
Female	815	51

	GO (n=420)		SGO (n=231)		RASIG (n=947)	
	N Male	**N Female**	**N Male**	**N Female**	**N Male**	**N Female**
Gender distribution for age classes						
60-64	72	83	41	54	170	162
65-69	72	104	59	38	155	164
70-74	35	47	27	4	143	147
75-79	0	7	7	1	2	4

Due to incompleteness of MARK-AGE database the number of subjects analyzed in this dissertation shifts between 1573 and 1598.

4.2.2 Socio-demographic characteristics

4.2.2.1 Marital status

From the marital status data obtained, most of the participants was married even though the RASIG showed the lowest percentage (68%). On the contrary RASIG population had a higher number of separate/divorced (13.9%) and widow/widower volunteers (12.8). Never married share was higher in GO participants. Separating data according gender, the number of married females was lower than males while the number of never married, separated/divorced or widow females (GO and RASIG) was higher as expected (Table 9).

Table 9 Marital status divided for groups and gender

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Marital status						
Never married	32	7.6	4	1.7	50	5.3
Married	316	75.2	220	95.2	644	68
Separated/Divorced	38	9.1	3	1.4	132	13.9
Widow/widower	34	8.1	4	1.7	121	12.8

	MALES			FEMALES		
Marital status per gender						
	GO %	**SGO %**	**RASIG %**	**GO %**	**SGO %**	**RASIG %**
Never married	6.2	1.5	2.8	8.8	1	7.8
Married	84.9	96.3	82.7	68	94.9	53.4
Separated/Divorced	5.6	1.5	9.8	11.6	1	18
Widow/widower	3.3	1.5	4.7	11.6	2	20.7

4.2.2.2 Level and years of education

The level of literacy among the volunteers was high and almost the totality of the sample received a formal education (only 0.4% of RASIG subjects never went to school and less of 2% of volunteers didn't complete elementary school level) even though on a different level. Examining the number of years spent in school the percentages are quite similar between

males. SGO and RASIG females reported a slightly lower amount of schooling years than GO females whose values were comparable with males. (Table. 10)

Table 10 Education level Education level and years of schooling divided for groups and gender

	MALES			FEMALES		
	GO Mean ± DS	**SGO Mean ± DS**	**RASIG Mean ± DS**	**GO Mean ± DS**	**SGO Mean ± DS**	**RASIG Mean ± DS**
Education: number of years	12.8 ± 5.2	12.8 ± 5.1	12.9 ± 4.3	12.6 ± 5	11.1 ± 5.0	11.7 ± 4.1
	GO %	**SGO %**	**RASIG %**	**GO %**	**SGO %**	**RASIG %**
Education level						
Never went to school	0	0	0	0	0	0.8
Did not finish elementary school	0	1.5	1.5	0	0	1.7
Finished elementary school	10.1	6.7	12.8	9.1	18.5	19
First stage of secondary level education	27.4	23.9	11.7	26.1	28.9	16.1
Second stage of secondary level education	20.7	21.6	22.5	22.4	23.7	19.1
Third level education other than university degree	12.9	17.2	13.8	17.4	13.4	22.6
Initial university degree or recognized equivalent	10.1	5.2	18.1	7.1	4.1	8.6
Higher university degree or post graduate	17.9	22.4	19.6	17	11.3	11.3
Do not know	1.1	1.5	0	0.8	0	0.6

4.2.2.3 Occupation

Great part of the sample declared to have an occupation during his life (96.7% GO, 92.9% SGO and 95.3% RASIG) (Table.11). The majority of subjects belonged to "**White Collars"** category with percentages very similar (shifting between 75.6% and 80%). The number of housekeepers was very low with the highest percentage in RASIG volunteers (6.2%). Considering the gender, the percentage of employed males subjects resulted higher (100% GO, 99.2% SGO and RASIG 99.6%) than females (94.2% GO, 93.8% SGO and only 1.2% RASIG). **"White collars"** occupation was predominantly in females volunteers even though in SGO females the percentage of **"Blue Collars"** occupation was higher than in the other

groups. Moreover, as expected, a much higher percentage of housekeepers was present among females and in particular in RASIG females.

Table 11 Main occupation classification divided for groups and gender

	GO		SGO		RASIG	
	N	%	N	N	%	N
White collars	336	80	174	75.6	745	78.8
Blue collars	67	16	51	22.2	141	15
Housewives	17	4	5	2.2	59	6.2

	MALES			FEMALES		
	GO %	SGO %	RASIG %	GO %	SGO %	RASIG %
White collars	74.3	75.4	79.6	84.2	76.2	78
Blue collars	25.1	23.9	18.9	9.2	21.5	11
Housewives	0.6	0.7	1.5	6.6	4.1	11

4.2.2.4 Living condition

The majority of the interviewed subjects lived in a private residence, house (included town house), farm or apartment (97.4% GO, 96.5% SGO, 99.4% RASIG) in comparable percentages for males and females. Very small indeed was the amount of the participants living in special dwelling for elderly or other kind of abodes. No subjects lived in nursing homes or residential care. (Table 12)

Moreover considering the cohabitation aspect, the majority of volunteers shared their domicile with their spouse, offspring, other relatives or friends (83.6% GO, 98.3 SGO, 76.4% RASIG). Noteworthy was the difference between the number of GO and RASIG subjects who lived alone (16.4% versus 23.6%). Regarding this point, splitting the data according to gender, the differences between GO and RASIG group were evident in males (7.2% GO, RASIG 12.8%) and even more noticeable in females (23.2 GO and 34.4 RASIG).

Table 12 Living condition – Housing and cohabitation divided for groups and gender

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Housing						
House, farm, apartment	409	97.4	223	96.5	943	99.4
Special dwelling for elderly	0	0	1	0.4	2	0.2
Nursing home	0	0	0	0	2	0.2
Other	11	2.6	7	3	3	0.3
Cohabitation						
With others	351	83.6	227	98.3	723	76.4
Alone	69	16.4	4	1.7	224	23.6

	MALES			FEMALES		
	GO %	**SGO %**	**RASIG %**	**GO %**	**SGO %**	**RASIG %**
Housing						
House, farm, apartment	97.8	95.5	99.8	97.1	97.9	99.2
Special dwelling for elderly	0	0.7	0	0	0	0.4
Nursing home	0	0	0	0	0	0
Other	2.2	3.7	0.2	2.9	4.1	0.4
Cohabitation						
With others	92.8	98.5	87.2	76.8	97.9	65.6
Alone	7.2	1.5	12.8	23.2	2.1	34.4

4.2.3 Lifestyle Factors

About half of the examined samples never smoked (48.6% GO, 45.9% SGO, 51.6% RASIG) while the other half consisted mostly in former smokers with a small quota of subjects who were currently smoking (Table 13). Only one-third of the sample was currently drinking alcohol every day (wine, beer or other alcoholic beverages) with comparable percentages between groups. The amount of total alcohol intake wasn't considered.

Table 13 Lifestyle habits divided into groups

	GO		SGO		RASIG	
	N	%	N	%	N	%
Smoking						
Never smokers	204	48.6	106	45.9	489	51.6
Former smokers	171	40.7	105	45.4	345	36.4
Smokers	45	10.7	20	8.7	113	12
Drinking						
Every day (wine, beer etc.)	129	30.7	73	31.6	286	30.2

4.2.4 Health Status

4.2.4.1 Health and morbidity

Analysing data referred to present pathologies, sensorial deficits were surely the most recurrent health problems among the interviewed volunteers and in particular vision impairment was the most frequent in the three groups: (48.3% GO, 42% SGO, 71% RASIG)

Table 14a Health status and morbidity distribution between groups

	GO		SGO		RASIG		P values
	N	%	N	%	N	%	
Present diseases							
Vision impairment	201	48.3	95	42	673	71	0.000500
Hearing impairment	72	17.4	35	15.3	201	21.3	0.047476
Neurological diseases	12	2.9	5	2.2	25	2.6	0.908546
Angina pectoris	15	3.6	21	9.2	61	6.5	0.012494
Irregular heart beat	31	7.5	33	14.5	120	12.7	0.003998
Heart failure	12	2.9	14	6.2	49	5.2	0.096952
High blood pressure	111	26.7	71	31.4	388	41	0.000500
Venous insufficiency in legs	54	12.9	24	10.4	122	12.9	0.607696
Pain in leg during walking	51	12.2	30	13	156	16.5	0.084958
Chronic respiratory diseases	32	7.6	13	5.6	68	7.2	0.676662
Chronic renal failure	2	0.5	1	0.5	7	0.7	0.913543
High cholesterol	111	26.7	76	32.9	348	37	0.001000
Diabetes	24	5.7	16	7	73	7.7	0.444278
Hyperthyroidism	11	2.6	4	1.8	28	3	0.648676
Hypothyroidism	29	6.9	13	5.7	109	11.6	0.001999
Autoimmune Diseases	7	1.7	6	2.6	47	5	0.006997
Cirrhosis of the liver	2	0.5	0	0	2	0.2	0.636182
Osteoporosis	40	9.6	18	7.9	127	13.5	0.017491
Arthritis	124	29.7	61	26.7	268	28.3	0.725137
Serious memory impairments	3	0.7	0	0	12	1.3	0.219890
Other mental health problems	11	2.6	8	3.5	25	2.6	0.764618

Performing a logistic regression is clearly visible that the impairment present in RASIG volunteers was extremely significant and age independent (Table 14b). The statistical model is constructed in order to predict the probability of development of pathology. Through logistic regression is possible to highlight the difference between RASIG and GO and also between SGO and GO.

Table 14b Vision impairment logistic regression corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-1.135547	0.178	0.321246
Gender[T.M]	-0.250503	0.0196	0.778409
Subject_Group[T.RASIG]	0.974715	**1.83e-15**	2.650412
Subject_Group[T.SGO]	-0.210161	0.211	0.810454
Age	0.017769	0.161	1.017927

Hearing impairment was less represented but also in this case RASIG group was the most affected (21.3%). Females resulted to be more compromised than males.

Table 15 Health status and morbidity per gender and groups

	MALES				FEMALES			
	GO %	**SGO %**	**RASIG %**	**P values**	**GO %**	**SGO %**	**RASIG %**	**P values**
Present diseases								
Vision impairment	42.6	39.4	69.3	0.000500	52.5	45.7	72.7	0.000500
Hearing impairment	19.4	20.1	23.8	0.361819	15.9	8.4	18.7	0.036982
Neurological diseases	3.3	3.7	3.4	0.966017	2.5	0	1.9	0.327336
Angina pectoris	3.9	13.5	8.4	0.006997	3.3	3.1	4.4	0.817091
Irregular heart beat	7.3	17.3	11.5	0.028986	7.6	10.5	13.8	0.040480
Heart failure	3.3	10.6	5.7	0.037981	2.55	0	4.6	0.041979
High blood pressure	27.5	30.3	41.3	0.001000	25.9	33	39.9	0.001000
Venous insufficiency in legs	8.9	5.2	6.8	0.452274	27.1	17.5	18.9	0.618191
Pain in leg during walking	10.7	15	13	0.519740	13.4	10.3	20	0.018491
Chronic respiratory diseases	7.9	8.9	5.3	0.202899	7.5	1	9	0.008996
Chronic renal failure	1.1	0.7	0.6	0.848076	0	0	0.8	0.431784
High cholesterol	24.1	32.1	33.7	0.063968	28.4	34	40.2	0.008496
Diabetes	7.3	6.7	7.7	0.982009	4.6	7.3	7.8	0.260370
Hyperthyroidism	0	0.8	2.1	0.073963	4.6	3.1	3.8	0.852074
Hypothyroidism	5.6	2.3	4.3	0.381309	7.9	10.3	18.8	0.000500
Autoimmune Diseases	1.1	3	4.3	0.117441	2.1	2.1	5.7	0.048976
Cirrhosis of the liver	0.6	0	0.2	0.645677	0.4	0	0.2	1.000000
Osteoporosis	2.2	0.8	2.1	0.660170	15	17.5	24.6	0.005997
Arthritis	23.6	21.2	21.9	0.866067	34.1	34	34.7	0.993003
Serious memory impairments	0.6	0	1.5	0.410295	0.8	0	1.1	0.873063
Other mental health problems	1.7	3	1.7	0.536732	3.3	4.2	3.6	0.932534

Regarding the presence of cardiovascular diseases GO group seemed to be better protected against arrhythmia, angina pectoris and heart failure since it showed the lower prevalence percentages.

Analysing data considering gender SGO males situation is surely noteworthy since they presented the higher values for arrhythmia, angina pectoris and heart failure (table 15).

Clusterizing reported cardiovascular pathologies (angina pectoris, hypertension, irregular heartbeat, heart failure) and performing a logistic regression, RASIG subjects proved to have the worst cardiovascular status and it tends to worsen with age with a very high relative risk (Table 15b).

Table 15b Cardiovascular cluster logistic regression corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-5.696023	4.3e-12	0.003359
Gender[T.M]	0.123640	0.233	1.131608
Subject_Group[T.RASIG]	0.590077	**1.92e-06**	1.804128
Subject_Group[T.SGO]	0.397393	0.0211	1.487941
Age	0.075362	**8.15e-10**	1.078275

Adding the presence of a previous myocardial infarction to the cardiovascular cluster RASIG situation worsen with values more significant than before.

Table 15c Cardiovascular cluster plus previous infarction logistic regression corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-5.600465	9.67e-12	0.003696
Gender[T.M]	0.145990	0.159	1.157185
Subject_Group[T.RASIG]	0.610602	**8.34e-07**	1.841539
Subject_Group[T.SGO]	0.408837	0.0175	1.505066
Age	0.073818	**1.77e-09**	1.076611

Moreover with regard to risk factors predisposing to cardiovascular diseases or that can aggravate their progression such as hypertension, hypercholesterolemia and diabetes, the first

two were very frequent among all participants and mainly in RASIG volunteers (41%), while diabetes was less represented but also in this case RASIG percentage was higher than in the other groups.

Clusterizing reported hypertension, hypercholesterolemia and diabetes and performing a logistic regression RASIG confirmed their difficulties and the situation is worsened by age (Table 15d).

Table 15d Metabolic cluster logistic regression corrected for corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-3.678481	6.94e-06	0.025261
Gender[T.M]	-0.060699	0.557	0.941107
Subject_Group[T.RASIG]	0.587252	**1.02e-06**	1.799038
Subject_Group[T.SGO]	0.343320	0.0406	1.409620
Age	0.053608	**1.26e-05**	1.055071

Muscle-articular apparatus disorders were reported by almost one-third of volunteers and females belonging to all groups reported the highest percentages (table 15). Concerning the presence of osteoporosis on the whole sample, only a small percentage of subjects was aware of being affected and RASIG group seemed to be more afflicted; as expected females volunteers (without distinction of group) had the highest prevalence. The past history of hip fracture, which correlates with osteoporosis, was present in a very limited number of subjects. Clusterizing reported articular pathologies (osteoporosis, arthritis and back-pain) and performing a logistic regression is confirmed that females has the worst condition

Table 15e Articular cluster logistic regression corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-2.315233	0.00443	0.098743
Gender[T.M]	-0.866171	**6.96e-17**	0.420559
Subject_Group[T.RASIG]	0.107460	0.376	1.113446
Subject_Group[T.SGO]	-0.108760	0.523	0.896946
Age	0.039691	0.00118	1.040489

The presence of chronic respiratory diseases such as COPD, asthma or chronic bronchitis is reported by a small number of candidates with percentages ranged between 5.6% and 7.2%. SGO females seemed to be the less affected (only 1%). The prevalence of leg venous insufficiency was comparable between groups but focusing on gender it appeared to be prerogative of females, as expected, and especially GO females (27.1%). Regarding the manifestation of leg pain during walking also known as Peripheral Artery Disease (PAD), a circulation problem in which the arteries in the legs become narrowed or blocked reducing the amount of oxygen that gets to the limbs, it seemed to affect mostly RASIG volunteers (16.5%) and in particular RASIG females (20%).

With regard to thyroid function, hyperthyroidism was scarcely present in all on the contrary hypothyroidism was reported in higher values and mostly by RASIG group. As expected, hyper and hypothyroidism affected mainly female volunteers and particularly RASIG females had the highest hypothyroidism prevalence.

Examining data referred to pathologies occurred in the past (Table 16), about one-fifth of the sample, with values ranging between 15.6% (GO) and 20.3% (SGO), reported at least one episode of pneumonia but this raw data does not differentiate between who contracted it at young age and who contracted it at older age.

Table 16 Health status and morbidity

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Past diseases						
Pneumonia	65	15.6	47	20.3	173	18.3
Myocardial infarction	8	1.9	13	5.7	40	4.2
Stroke, cerebral thrombosis	15	3.6	9	3.9	30	3.2
Malignant Tumour	16	3.8	10	4.3	29	3.1
Hip fracture	9	2.2	3	1.3	16	1.7
Other not mentioned health problems	97	23.1	48	20.9	247	26.2
Use of prescribed medicines	278	66.7	160	70.2	693	73.2
Hospitalization in the last year	49	11.7	30	13	137	14.5
Falls in the last year	56	13.5	27	11.7	117	12.4
Loss of weight in the last year	139	33.17	74	32	323	34.2

	MALES			FEMALES		
	GO %	SGO %	RASIG %	GO %	SGO %	RASIG %
Past diseases						
Pneumonia	18	24.6	17.5	13.8	14.4	19.1
Myocardial infarction	2.8	9	6.8	0.8	1	1.6
Stroke, cerebral thrombosis	4	5.3	2.8	3.3	2.1	3.6
Malignant Tumour	7.3	9.7	7.5	12	8.2	7
Hip fracture	2.8	1.5	1.1	1.6	1	2.3
Other not mentioned health problems	25.1	26.1	25.1	21.6	13.6	27.3
Use of prescribed medicines	63.1	70.7	68.1	68.9	69.5	78.2
Hospitalization in the last year	13.4	15.7	15.8	10.4	9.3	13.3
Falls in the last year	10.7	8.2	8.1	15.5	16.5	16.6
Loss of weight in the last year	27.9	29.1	30.1	37.1	36.1	38.3

The prevalence of previous myocardial infarction was quite low among volunteers and GO seemed to be more protected than SGO and RASIG (Table 16) but taking into account gender, it is important to point out that actually all females, without distinction, were less affected than males.

Performing a logistic regression on previous myocardial infarction data, as expected, males were the most affected (Table 16b).

Table 16b Previous myocardial infarction logistic regression corrected for groups, gender and age

	coef	pvalues	relative risk
Intercept	-8.649374	3.49e-05	0.000175
Gender[T.M]	1.517001	**7.8e-06**	4.558533
Subject_Group[T.RASIG]	0.812315	0.0518	2.253118
Subject_Group[T.SGO]	1.030882	0.0323	2.803537
Age	0.055239	0.0712	1.056793

The occurrence of stroke and cerebral thrombosis was comparable between groups and gender. Regarding previous cancer, GO females reported the highest prevalence. Noteworthy was the number of volunteers (more than one-fifth of the sample) declaring to have other

medical conditions in addition to those investigated (23.1% GO, 20.9% SGO, 26.2% RASIG). Unexpectedly, only 13.6% of SGO females reported additional pathologies.

As harbingers of incipient decay of the physical performance the presence of three events occurred in the last 12 months were investigated:

1. Number of falls
2. Hospitalization (with an overnight stay)
3. Loss of weigh

Concerning the falls no significant differences were found since volunteers showed comparable percentages (13.5% GO, 11.7% SGO, 12.4% RASIG) even though females reported higher prevalence (without distinction of group). The percentage of volunteers subjected to hospitalization shifted between 11.7% (GO) and 14.5% (RASIG) and the number of hospitalized males was higher than females. Concerning weight, data showed that one-third of the sample referred loss of weight in the last year in comparable percentages among groups while females showed the highest percentages. Unfortunately it was not reported if the loss of weight was intentional (e.g. due to diet or physical exercise) or not.

An assessment of health status at the time of the interview, described on the basis of the presence of disease and use of drugs was performed.

Multimorbidity or the presence of two or more medical conditions within the same individual is an extremely recurrent event in the elderly. For this reason the number of pathologies reported by volunteers was analysed (Table 17). The number of pathologies ranged between 2.5 (GO) and 4.2 (RASIG) per capita. Data pointed out that RASIG subjects were significantly more affected than the other groups while no substantial difference was found between GO and SGO. Considering gender, in all three groups females showed a higher and significant morbidity. As expected, age played an important role (Table 18).

Table 17 Number of pathologies divided into groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	179	2.519553	1.972681	0	1	2	4	8
	F	241	3.161826	2.139596	0	2	3	4	11
SGO	**M**	134	2.798507	2.386616	0	1	2	4	12
	F	97	3.072165	2.416296	0	1	3	5	10
RASIG	**M**	470	3.206383	2.064514	0	2	3	4	11
	F	477	4.215933	2.497376	0	2	4	6	17

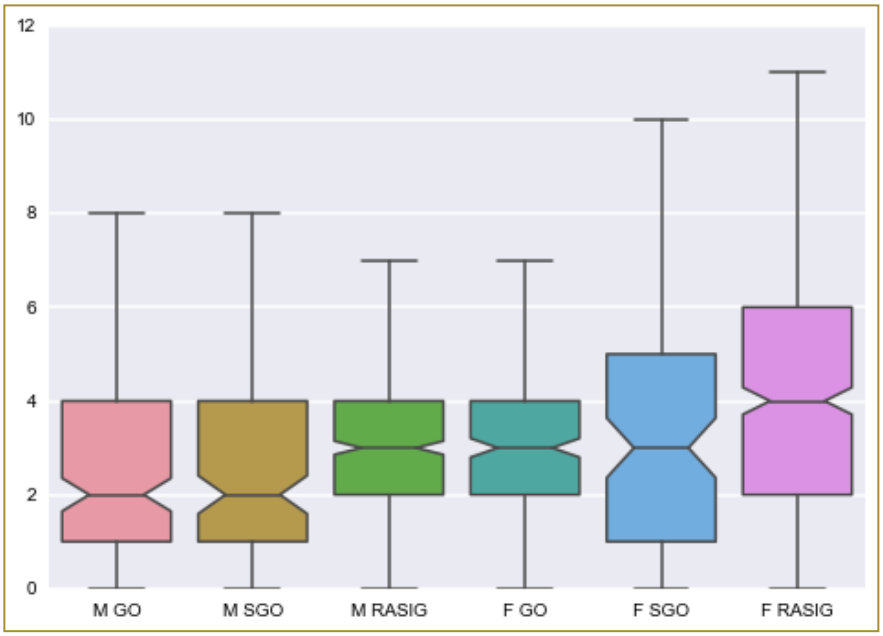


Fig. 19 Distribution of the number of pathologies in groups and gender

Table 18 Linear regression of number of pathologies

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-3.143558	0.000236	-4.819413	-1.467702
Subject_Group[T.RASIG]	0.742504	**6.24e-09**	0.492030	0.992979
Subject_Group[T.SGO]	0.048794	0.785	-0.300994	0.398581
Gender[T.M]	-0.777255	**1.02e-12**	-0.990978	-0.563531
Age	0.094836	**1.51e-13**	0.069672	0.120001

The relation between number of pathologies and age was investigated in order to estimate the effect of age on the three different groups of volunteers (Table 19). To better address the problem the whole RASIG population (younger volunteers included) was considered.

Table 19 Number of pathologies and age linear regression corrected for group and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-2.066621	4.22e-19	-2.520146	-1.613097
Subject_Group[T.RASIG]	0.625992	**6.94e-11**	0.437867	0.814116
Subject_Group[T.SGO]	0.018699	0.891	-0.248242	0.285640
Gender[T.M]	-0.628531	**4.85e-20**	-0.762912	-0.494151
Age	0.079028	**2.63e-121**	0.072414	0.085641

As expected age had a tremendous and significant impact on multimorbidity especially on females as reported in literature. As seen before, RASIG population showed a significant worse situation with respect to GO and SGO who, on the contrary displayed no significant differences (Table 19). In fact, observing the distribution of morbidities in the three groups, it is clearly evident an increase with age apparently more strong in RASIG (Fig. 20).

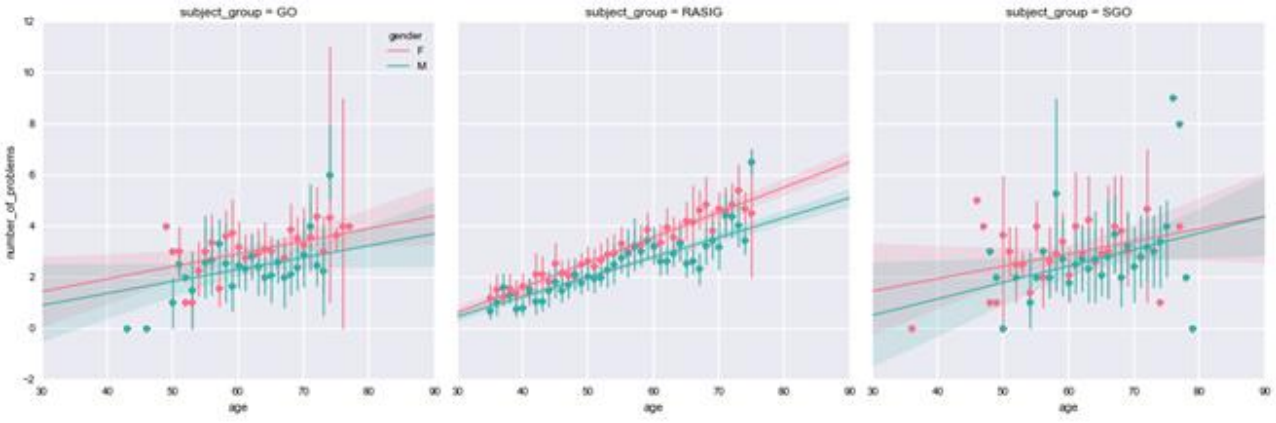


Fig. 20 Number of pathologies linear regression

Since medication use in the elderly is an important issue, the percentage of volunteers reporting use of prescribed medicines was considered. The percentages found were very high spanning from 66.7% of GO to 70.2% of GO up to an impressive 73.2% of RASIG who also in this occasion were confirmed as the subjects in the worst situation. Considering that poly-

pharmacy is increasing among older people the number of medications used was analysed (Table 20).

Table 20 Number of medicines used divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	179	1.765363	2.069231	0	0	1	3	9
	F	241	1.804979	2.010218	0	0	1	3	10
SGO	**M**	134	2.320896	2.363891	0	0	2	4	10
	F	97	2.000000	2.005202	0	0	2	3	9
RASIG	**M**	470	2.104255	2.217344	0	1	2	4	10
	F	477	2.597484	2.363375	0	0	2	3	10

The analysis showed that the number of medications used ranged between 1.76 (GO) and 2.59 (RASIG) per capita. RASIG subjects showed a worse situation and in particular RASIG females who reported the highest numbers of medicine used (Fig. 21).

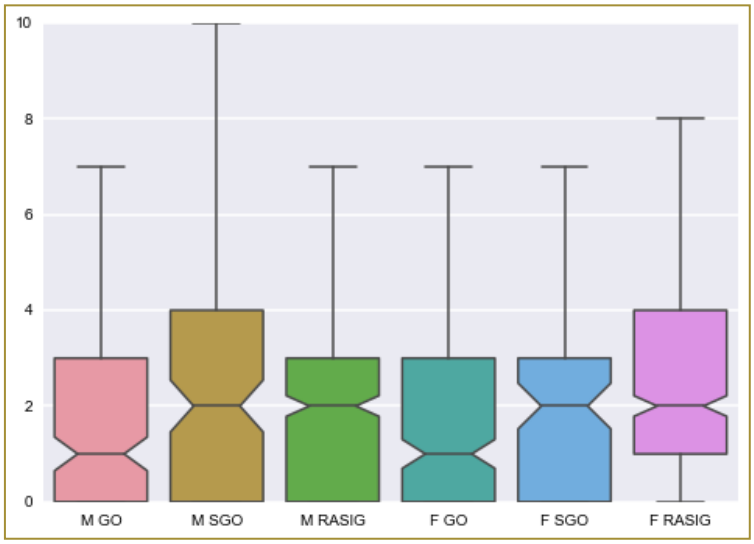


Fig. 21 Distribution of number of medicines used divided for groups and gender

As previously done with pathologies, the correlation between number of medications used and age was examined (Table 21). Also in this case, as predicted, age showed a crucial impact but unexpectedly no significant difference was found between males and females. RASIG group confirmed, also in this occasion, to be the group with more difficulties (Fig. 22).

Table 21 Number of medicines and age linear regression corrected for group and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-4.567761	5.27e-09	-6.101195	-3.034327
Subject_Group[T.RASIG]	0.463646	**7.34e-05**	0.234458	0.692834
Subject_Group[T.SGO]	0.455377	0.00529	0.135316	0.775438
Gender[T.M]	-0.269686	0.00687	-0.465246	-0.074125
Age	0.095165	**5.48e-16**	0.072139	0.118191

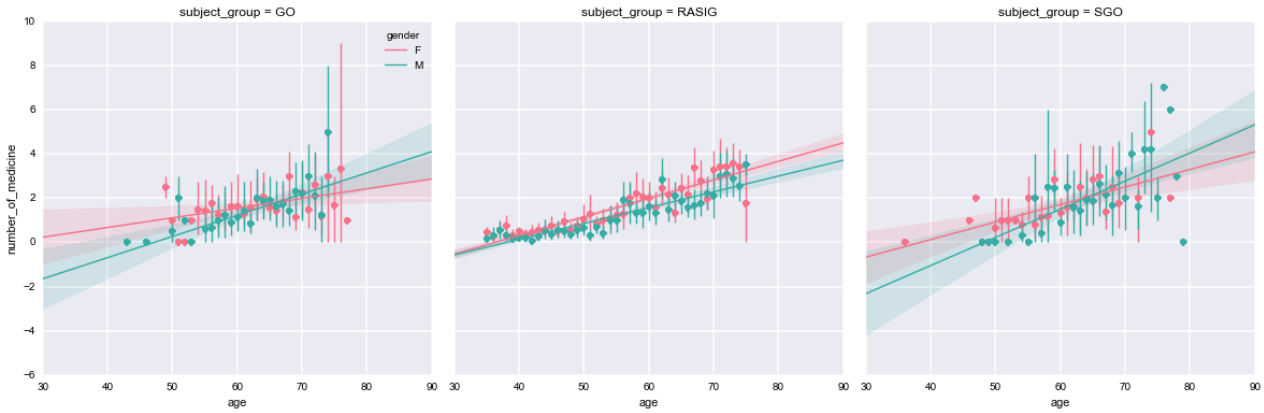


Fig. 22 Number of medicines and age linear regression

All the clinical parameters examined so far were self-reported and this could suggest that the prevalence of certain diseases could be higher than registered because the subjects may be not aware of being affected. In order to test this hypothesis two parameters as hypercholesterolemia and diabetes were explored. For this analysis self-reported prevalence, specific medication use and serum cholesterol level >6.2 mmol/l (for hypercholesterolemia) and serum glucose level > 6.9 mmol/l (for diabetes) were considered (Fig. 23). The glucose and cholesterol serum level was included in the evaluation in order to found undetected cases of metabolic diseases. The test was performed on the whole GO, SGO and RASIG population combined (considering all age classes) and it showed clearly that, besides an expected age related increase of total prevalence, very considerable was the quota of subjects that appears to be affected by metabolic pathologies without being aware of (Fig. 23).

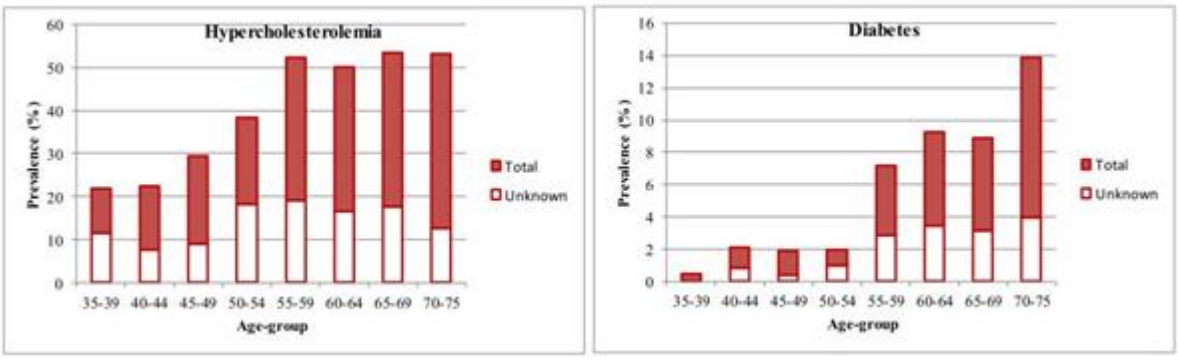


Fig.23 Total prevalence of hypercholesterolemia and diabetes by age classes in GO, SGO and RASIG volunteers. The white colour indicates the unknown prevalence based only on serum levels.

4.2.4.2 Self-reported health

It is well known in literature that self-reported health status is a good predictor of forthcoming disability, hospitalization and mortality (Idler et al. 1995, Idler et al. 1997, Gold et al. 1996, Miilunpalo et al. 1997, Jamoom et al. 2008). It was estimated the level of perceived health status with the following question from SF-36v1 Health Survey: *"In general, would you say your health is"*. The percentages of five-choice response scale (excellent, very good, good, fair and poor) were considered for the analysis.

Almost one-third of the subjects judged to have a "very good" health status (SGO had the lower percentage) while more or less half of the sample and in particular SGO volunteers considered their health as "good" (Table 22). Considering the extremes of the scale, the "excellent" and the "poor" health status, GO subjects reported the highest percentage of "well-being" (14.5%) declared as "excellent" health status and the lowest percentage of "illness" (0.5%) considered as "poor". On the contrary RASIG volunteers reported the lowest percentage of "well-being" (9.2%) while SGO reported the worst health status. Considering the gender variable, the "excellent" health status was reported mainly by males subjects (with GO in the frontline) compared to females (in particular RASIG females). The percentages of "poor" health status of GO were the lowest (males and females) while were the highest into SGO and RASIG males.

The five-choice response scale was clustered in two groups:

1. **"Healthy"** including those who considered their health status good, very good and excellent.
2. **"Not healthy"** including those who considered their health status fair and poor.

Table 22 Self-reported Health Status

	GO		SGO		RASIG	
	N	%	N	%	N	%
Self-reported health						
Excellent	61	14.5	23	10	87	9.2
Very good	123	29.3	54	23.4	305	32.2
Good	177	42.1	111	48	397	42
Fair	57	13.6	35	15.1	136	14.3
Poor	2	0.5	8	3.5	22	2.3
Self-reported health						
Healthy	361	86	188	81.3	789	83.3
Not healthy	59	14	43	18.7	158	16.7

	MALES			FEMALES		
	GO %	SGO %	RASIG %	GO %	SGO %	RASIG %
Self-reported health						
Excellent	17.3	10.4	10.8	12.5	9.3	7.5
Very good	28.5	23.9	34	29.5	22.7	30.4
Good	39.1	47	40.8	44.5	49.5	43
Fair	14.5	14.2	11.5	12.9	16.5	17.2
Poor	0.6	4.5	2.8	0.5	2	1.9

The majority of GO volunteers (86%) were convinced to have a "healthy" status compared to RASIG (83.3%) and SGO subjects (81.3%). Considering the number of subjects who had perception of "poor" health status SGO volunteers had the highest percentages (Table 22).

The possible correlation between perceived health status and age was explored and no significant data was found (Table 23).

Table 23 healthy" volunteers and age logistic regression corrected for group and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	4.182376	8.98e-05	2.089364	6.275388
Subject_Group[T.RASIG]	-0.185332	0.266	-0.511610	0.140945
Subject_Group[T.SGO]	-0.376122	0.0896	-0.810353	0.058110
Gender[T.M]	0.186734	0.174	-0.082189	0.455658
Age	-0.036929	0.0204	-0.068151	-0.005708

4.2.5 Disability

4.2.5.1 ADL

Aging process is very frequently characterized by disability. The level of disability was evaluated with Katz ADL Scale (continence excluded) and the obtained results were categorized with a three level scale where the volunteers were classified as not impaired, moderately impaired and severely impaired (Table 24). Almost the entire sample was completely independent with comparable data between the three groups. Considering gender, very small percentages of GO males (1.1%) and SGO females (1%) showed to be severely impaired. The differences found between groups, males and females were no significant.

Table 24 ADL scale

	MALES			FEMALES		
	GO %	**SGO %**	**RASIG %**	**GO %**	**SGO %**	**RASIG %**
ADL						
Severely impaired (ADL=0-2)	1.1	0	0	0.4	1	0
Moderately impaired (ADL=3-4)	0.6	0.7	0.2	0.4	1	0
Not impaired (ADL=5)	98.3	99.3	99.8	99.2	98	100

A said before, the continence item from Katz's Scale was considered separately from the others items (Table 25).

Incontinence problems were higher in GO volunteers (17.7%) than in SGO (14.8%) and RASIG (13.2%) however no significant difference was found. Analysing data considering gender, as expected, incontinence was a female prerogative in fact the problem was present in 22.5% of females against 6.5% of males. In this case a significant difference was found.

Table 25 Evaluation of continence

	GO		SGO		RASIG		pvalues
	N	%	N	%	N	%	
Continence							
Leak urine	74	17.7	34	14.8	117	13.2	0.09295
Full continence	343	82.3	196	85.2	772	86.8	
	MALES				**FEMALES**		
Leak urine	6.5				22.5		**2.2e-16**
Full continence	93.5				77.5		

4.2.5.2 Nagi-scheme

The analysis of disability was enriched with the evaluation of several questions from the Nagi-scheme (Nagi SZ, 1976) evaluating the sensorial and physical abilities.

Almost one-fifth of the sample was able to read newspaper without glasses even though SGO (17%) showed to have more difficulty than GO (20.2%) and RASIG (21.6%) and a very high number of subjects was able to recognize someone 4 m far away without glasses (Table 26). Also in this case SGO (81.2%) had more difficulty than GO (86.6%) and RASIG (86%). Nearly the entire sample had no difficulties in hearing without aids with comparable percentages among groups.

Regarding functional status all the sample seemed to be completely independent and without particular difficulties (Table 26). The percentages of subjects able to walk for about 500 m without aids ranged between 97.1% (GO) and 99.6% (RASIG) The same applied for the ability of going up and down the stairs without anyone's help where 98.3 % of GO and SGO and 99.9of RASIG subjects showed no difficulties. Also the ability of doing any kind of light housework or exercise was completely conserved by almost all the sample (Table 26).

Table 26 Sensorial and functional status

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Nagi Scheme						
Reading newspaper without glasses	85	20.2	39	17	203	21.6
Recognize someone 4 m away without glasses	363	86.6	198	86	765	81.2
Hearing ability without aids	412	98.1	222	97	907	96.2
Functional status						
500 m walking ability without aids	406	97.1	228	99.6	930	98.7
Going up and down the stairs without anyone's help	411	98.3	225	98.3	941	99.9
Doing any kind of exercise	405	98.8	224	99.6	931	99.3

4.2.6 Functional status

The volunteers functional status was tested with Chair Stand Test and measuring the handgrip strength.

4.2.6.1 Chair Stand Test

Firstly was evaluated candidates ability to perform the exercise without problems. Subjects were divided in three categories: ability to perform the exercise without difficulties, necessity to stop after 1-4 times and not able at all to perform (Table 27).

The majority of the sample was able to complete test exercise without problems and only very few subjects had to stop before the test end. Very small percentages of GO (2.6%) and RASIG (2.7%) were not able at all to perform the exercise.

Table 27 Chair Stand Test

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Chair Stand Test						
Able to perform	408	97.2	229	99.2	918	97
Stop after 1-4 times	1	0.2	1	0.4	3	0.3
Not able at all	11	2.6	1	0.4	25	2.7

Only the volunteers able to complete the test were considered for the analysis and it was carried out on the number of seconds spent to complete 5 chair stands.

The average time spent by groups to perform the exercise was 10 seconds with the exception of RASIG males that used only 9.4 seconds (Table 28).

Table 28 Chair Stand Test results divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	173	10.416185	2.867252	5	8	10	12	23
	F	235	10.536170	2.621772	5	9	10	12	25
SGO	**M**	133	10.624060	3.401429	5	8	10	12	25
	F	96	10.645833	2.722148	6	9	10	12	23
RASIG	**M**	456	9.405702	2.948624	4	8	9	11	28
	F	462	10.253247	3.589382	4	8	10	11	54

As expected ages significantly affected the test performance of test and also gender played an important role (Table 29). RASIG subjects had the best performance (significant values).

Table 29 Chair Stand test and age linear regression corrected for group and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	5.463971	1.18e-07	3.442093	7.485849
Subject_Group[T.RASIG]	-0.799706	**1.9e-07**	-1.100597	-0.498814
Subject_Group[T.SGO]	0.200594	0.346	-0.216457	0.617644
Gender[T.M]	-0.543651	**3.22e-05**	-0.799957	-0.287345
Age	0.076368	**8.65e-07**	0.045947	0.106790

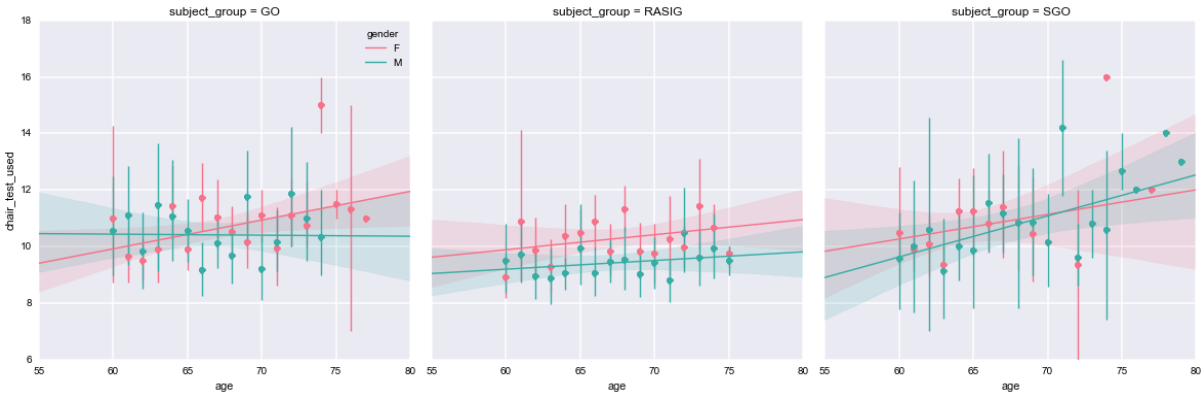


Fig. 24 Chair Stand test and age linear regression

4.2.6.2 Handgrip strength

For this evaluation only volunteers able to complete the test were considered. The exercise consisted in three performances with each hand. The best performance of the dominant hand (strength expressed in kilograms) was used for the analysis (Gueresi et al., 2013).

Males and females performed in a different way in accord with literature (Table 30). Males strength on average shifted between 40.9 Kg (RASIG) and 44.8 Kg (GO). Females strength on average ranged between 26.1 Kg (RASIG) and 27.5 Kg (GO).

Table 30 Hand grip strength divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	176	44.806818	9.951147	1	40	44.5	51	100
	F	232	27.573276	5.606410	14	24	27.0	32	42
SGO	**M**	131	42.664122	8.353360	22	37	43	48	66
	F	97	28.216495	5.381734	18	24	27	32	44
RASIG	**M**	461	40.965293	8.145343	0	35	41	46	62
	F	475	26.143158	5.108582	10	23	26	30	44

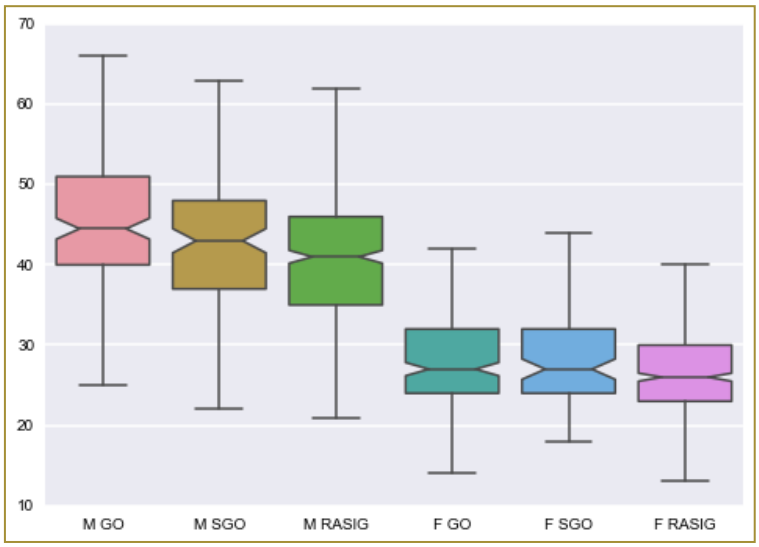


Fig. 25 Violin plot of hand grip strength divide for groups and gender

As happened before no particular difference was noted between GO and SGO subjects (both sexes) while a significant difference was found in RASIG volunteers (in particular RASIG males) that also in this case had a significant worse performance (Table 31). As predicted, age had a significant impact on the test and particularly on RASIG volunteers (Fig.26).

Table 31 hand grip strength and age linear regression

	coef	pvalues	[95\% Conf.	Int.]
Intercept	55.879941	7.83e-109	50.938233	60.821649
Subject_Group[T.RASIG]	-1.877921	**7.34e-07**	-2.621168	-1.134673
Subject_Group[T.SGO]	-0.779574	0.14	-1.814360	0.255211
Gender[T.M]	15.616611	**0**	14.985046	16.248177
Age	-0.420594	**1.2e-28**	-0.494833	-0.346356

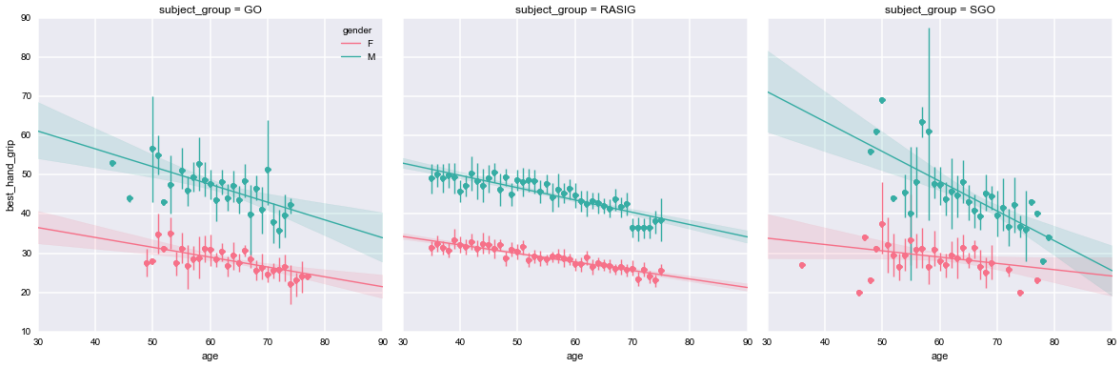


Fig. 26 Hand grip strength and age linear regression

4.2.7 Anthropometric measurements

In all recruited volunteers total height, knee height and the weight were measured. The data regarding knee height are not reported in this dissertation.

Concerning total height, results indicate, as expected, that males were taller than females and in particular GO males (Table 32). Males total height ranged between 173.3 cm ± 6.7 (s.d.) (RASIG) and 175.1 cm ± 8 (GO). Females total height shifted between 160.8 cm ± 6.3 (RASIG) and 162.9 cm ± 6.9 (SGO). In both sexes RASIG subjects were the smallest.

Weight, as for height, was higher in males than females. Males values were comprised between 81.4 Kg ± 11.6 (RASIG) and 85.2 Kg ± 13.4 (SGO) while females weight ranged between 68.8 Kg ± 12.2 (GO) and 71.7 Kg ± 12 (SGO).

Table 32 Anthropometric measurements

	MALES			FEMALES		
	GO Mean ± DS	**SGO Mean ± DS**	**RASIG Mean ± DS**	**GO Mean ± DS**	**SGO Mean ± DS**	**RASIG Mean ± DS**
Height	175.1 ± 8	175.7 ± 7.2	173.3 ± 6.7	162,7 ± 6.2	162.9 ± 6.9	160.8 ± 6.3
Weight	84.3 ± 12.8	85.2 ± 13.4	81.4 ± 11.6	68.8 ± 12.2	71.7 ± 12	70.1 ± 13.4
BMI	27.5 ± 3.8	27.6 ± 4	27.1 ± 3.5	26 ± 4.5	27.1 ± 4.9	27.2 ± 5.1

BMI (Body Mass Index) calculated on weight and height data (K/m^2) showed no particular differences between groups and gender and ranging between 26 ± 4.5 DS (GO females) and 27.5 ± 3.8 DS (GO males).

4.2.8 Cognitive Status

4.2.8.1 Mini-Mental State Examination (SMMSE)

SMMSE (Molloy et al., 1991) is the most commonly used instrument for screening cognitive function and can be used to indicate the presence of cognitive impairment.
The entire sample obtained very high comparable scores (in all three groups) and also in both sexes (Table 33). Males scored between 28.4 (SGO) and 28.8 (GO) and females between 28.7

(RASIG) and 29 (SGO). Analysing data for groups and gender no significant differences were found (Table 33)

Table 33 Mini-Mental State Examination (SMMSE) divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	177	28.887006	1.405630	23	28	29	30	30
	F	241	28.937759	1.338635	24	28	29	30	30
SGO	**M**	133	28.458647	1.621406	24	27	29	30	30
	F	97	29.020619	1.224569	24	29	29	30	30
RASIG	**M**	452	28.761062	1.415933	23	28	29	30	30
	F	464	28.709052	1.656013	20	28	29	30	30

All the participants were divided in three categories (Nybo et al., 2003) on the base of the obtained score:

1. “not impaired” (24–30 points)
2. “mild impaired” (18–23 points)
3. “severely impaired” (0–17 points)

As expected almost the entire sample was classified as “not impaired” along with a very, very scarce percentage of subjects classified “mild impaired” (0.2% of GO and 1.1% of RASIG) (Table 34). No severely impaired volunteers were found.

Table 34 Mini-Mental State Examination (SMMSE) classification

	GO		SGO		RASIG	
	N	**%**	**N**	**%**	**N**	**%**
Not impaired (24-30)	417	99.8	230	100	906	98.9
Mild Impaired (18-23)	1	0.2	0	0	10	1.1
Severely impaired (0-17)	0	0	0	0	0	0

As previously done, SMMSE test correlation with age was evaluated. No significant values were found.

Table 35 SMMSE and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	31.087456	0	29.895043	32.279870
Subject_Group[T.RASIG]	-0.087274	0.282	-0.246116	0.071568
Subject_Group[T.SGO]	-0.158043	0.165	-0.381391	0.065306
Gender[T.M]	-0.039296	0.57	-0.174927	0.096334
Age	-0.023062	0.00812	-0.040138	-0.005986

4.2.8.2 15 Picture Learning Test (15-PLT)

15 Picture Learning Test (Brand and Jolles, 1985) is used to assess the immediate and delayed memory function. The test consists in three consecutive and repeated trials and a postponed recall after 20 minutes. The sum of the number of correct remembered pictures in the first three trials and the number of correct remembered pictures after the pause were used for the analysis. Low scores indicate a cognitive decline. Only the participants able to complete the test were evaluated.

Sum of the three trials

The three groups scored similarly without particular differences. The number of remembered figures shifted between 29 (GO) and 32.3 (SGO) (Table 36).

Table 36 15-PLT (sum of 3 trials) divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	177	29.084746	5.962825	10	25	30	34	42
	F	237	32.021097	4.734875	18	29	32	36	43
SGO	**M**	131	29.083969	5.240432	14	26	30	32.5	41
	F	96	32.354167	4.462662	22	29	33	36	41
RASIG	**M**	453	30.048565	5.252671	11	27	30	34	43
	F	464	31.342672	5.698417	12	28	32	35	43

Considering the "gender" variable, females showed to be significantly more efficient in the performance obtaining higher scores in all three groups (Table 37)

As occurred previously, age impacted significantly on the execution of the test without significant differences in the three groups (Table 37) (Fig. 27)

Table 37 15-PLT (sum 3 thrials) and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	48.933423	1.08e-115	44.738540	53.128307
Subject_Group[T.RASIG]	0.382581	0.23	-0.242001	1.007163
Subject_Group[T.SGO]	-0.125344	0.778	-0.995854	0.745165
Gender[T.M]	-2.044050	**6.5e-14**	-2.578384	-1.509716
Age	-0.259449	**7.09e-16**	-0.322470	-0.196428

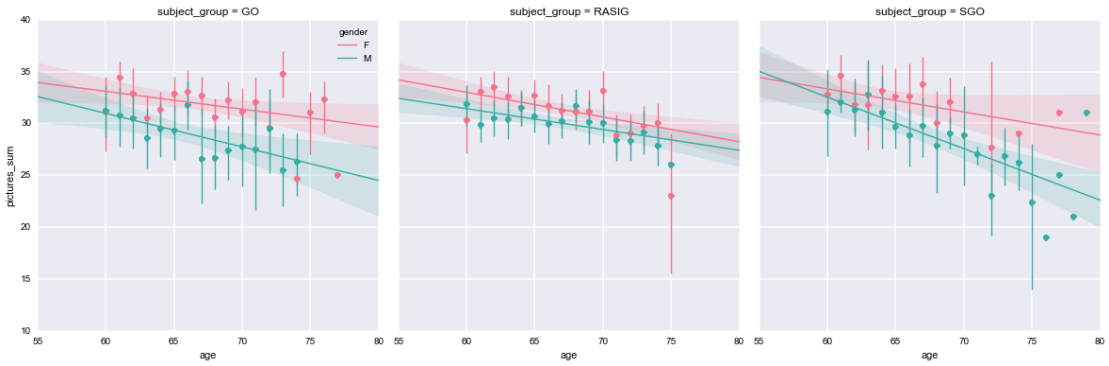


Fig. 27 15-PLT (sum 3 thrials) and age linear regression

Recall after 20 minutes

As intended by test execution protocol participants were required to recall the figures previously seen in the three trials.

Examined data showed that, as happened for the three trials, no particular differences were found in the three groups whose scores were comparable (Table 38)

Table 38 15-PLT recall divided for groups and gender divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	177	11.039548	2.691235	2	9	11	13	15
	F	237	12.101266	2.100817	4	11	12	14	15
SGO	**M**	131	11.030534	2.252994	5	10	11	13	15
	F	96	12.322917	2.013088	5	11	13	14	15
RASIG	**M**	453	11.379691	2.332292	4	10	12	13	15
	F	464	11.976293	2.394531	0	10	12	14	15

Also in this case a "gender effect" was noticed. In fact in the recall trial females had a better and significant performance too (Table39).

Table 39 15-PLT (recall) and age 15-PLT (sum 3 thrials) and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	18.864608	1.1e-95	17.083182	20.646035
Subject_Group[T.RASIG]	0.166438	0.219	-0.098801	0.431677
Subject_Group[T.SGO]	-0.042211	0.823	-0.411887	0.327465
Gender[T.M]	-0.825554	**9.98e-13**	-1.052468	-0.598641
Age	-0.101619	**9.92e-14**	-0.128382	-0.074856

Even in this case age impacted significantly on the execution of the test (Table 39) (Fig. 28)

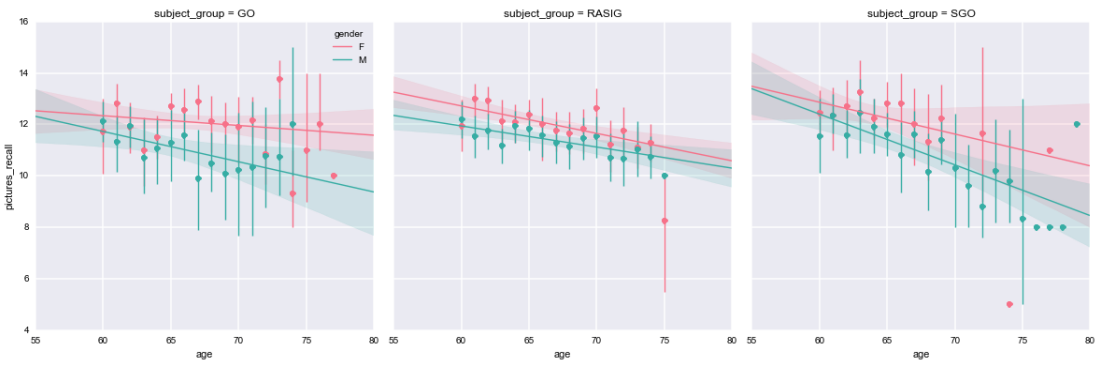


Fig. 28 15 Picture Learning Test recall and age linear regression

4.2.8.3 Stroop Colour Word Test

Stroop Test is used to test selective attention. The test consists in three sheets showing 40 stimuli each, which the subject was asked to read or name as quickly as possible. The number of seconds spent performing the tasks were considered for the analysis. Lower is the number of seconds used the better the performance.

Stroop Test Card 1 - Colour names

The time used to complete the first trial ranged between 19.5 seconds (RASIG) and 21.7 seconds (SGO) (Table 40). No particular variation between GO and SGO groups was noted on the contrary RASIG volunteers had the best significant performance (Table 41)

Table 40 Stroop Test Card 1 divided for groups and gender divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	177	21.463277	5.829008	13	18	20	24	63
	F	241	21.219917	5.243784	12	17	21	24	42
SGO	**M**	134	21.798507	5.950649	12	18	20	24	42
	F	97	20.886598	5.032967	12	18	20	23	44
RASIG	**M**	463	19.596112	7.144076	11	16	19	22	129
	F	463	20.205184	5.379436	11	16	19	23	45

Confirming expectations age showed to have an influence on the test performance (Fig. 29).

Table 41 Stroop Test Card 1 and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	11.329390	1.69e-09	7.643657	15.015124
Subject_Group[T.RASIG]	-1.641430	**4.12e-09**	-2.188626	-1.094235
Subject_Group[T.SGO]	0.075983	0.845	-0.685353	0.837319
Gender[T.M]	-0.405243	0.0899	-0.873517	0.063032
Age	0.145822	**2.45e-07**	0.090452	0.201191

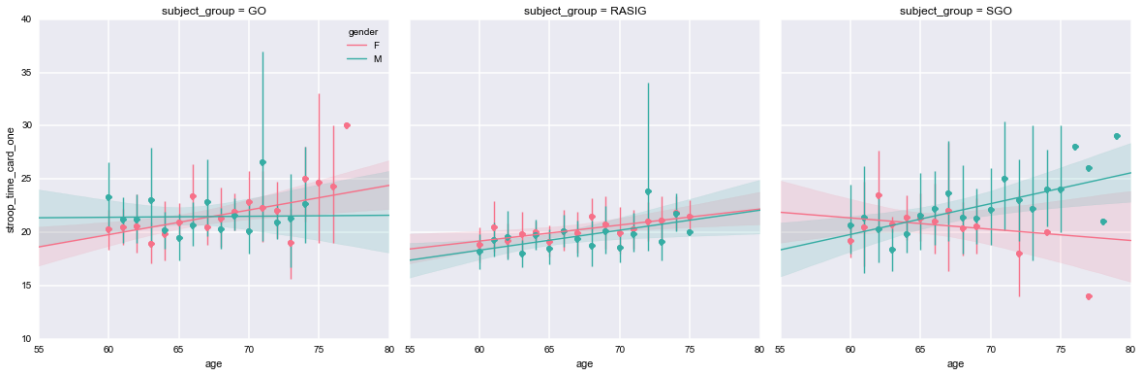


Fig. 29 Stroop Test Card 1 and age linear regresssion

Stroop Test Card 2 - Coloured patches

The time used to complete the second trial ranged between 26.6 seconds (SGO) and 28.7 seconds (SGO) (Table 42). No particular difference between groups was noted since times were comparable. No gender effect was found (Table 43).

Table 42 Stroop Test Card 2 divided for groups and gender divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	178	27.831461	7.976287	17	23	26	30	80
	F	240	27.041667	6.914173	17	22	25	30	36
SGO	**M**	134	28.776119	8.251354	17	23	27	32	63
	F	97	26.649485	6.245067	17	23	26	29	57
RASIG	**M**	464	26.711207	7.327174	15	22	25	30	54
	F	471	26.651805	6.955007	15	22	25	30	57

The only significant noteworthy data was the influence of age (Table 43).

Table 43 Stroop Test Card 2 and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	9.732499	3.24e-05	5.142313	14.322685
Subject_Group[T.RASIG]	-0.759481	0.0293	-1.442572	-0.076391
Subject_Group[T.SGO]	0.585165	0.228	-0.366586	1.536916
Gender[T.M]	0.195266	0.512	-0.388357	0.778888
Age	0.250099	**1.15e-12**	0.181164	0.319034

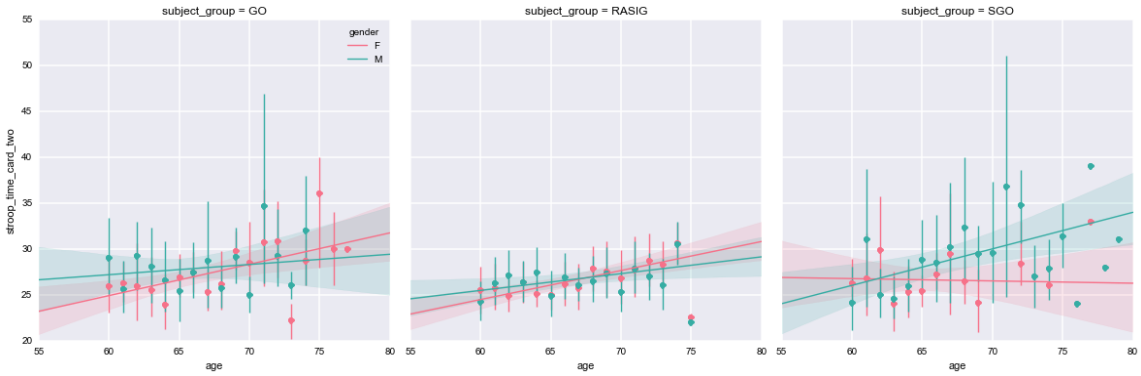


Fig. 30 Stroop Test Card 2and age linear regression

Stroop Test Card 3 - Colour names printed in incongruously coloured ink.

As expected, increasing the test difficulty the number of seconds spent in order to complete the trial increased. The times used ranged between 52.5 seconds (SGO) and 59.6 seconds (SGO) (Table 44).

Table 44 Stroop Test Card 3 divided for groups and gender divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	178	54.151685	22.659467	25	39	48	60	180
	F	239	53.263598	20.750052	26	40	48	60	137
SGO	**M**	133	59.669173	24.840315	21	43	52	69	170
	F	97	52.515464	18.720596	25	39	50	59	120
RASIG	**M**	463	53.224622	18.588308	25	41	48	60	173
	F	469	55.375267	20.252991	20	42	50	63	154

No particular trends were noted and no significant differences between groups and gender were found (Table 45)

Evaluating data obtained, age seemed to be the only significant relation (Table 45) (Fig. 31)

Table 45 Stroop Test Card 3 and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-0.952290	0.876	-12.906707	11.002127
Subject_Group[T.RASIG]	0.893595	0.325	-0.885542	2.672731
Subject_Group[T.SGO]	2.804334	0.0267	0.324574	5.284094
Gender[T.M]	-0.367248	0.636	-1.887580	1.153084
Age	0.773399	**3.15e-17**	0.593816	0.952982

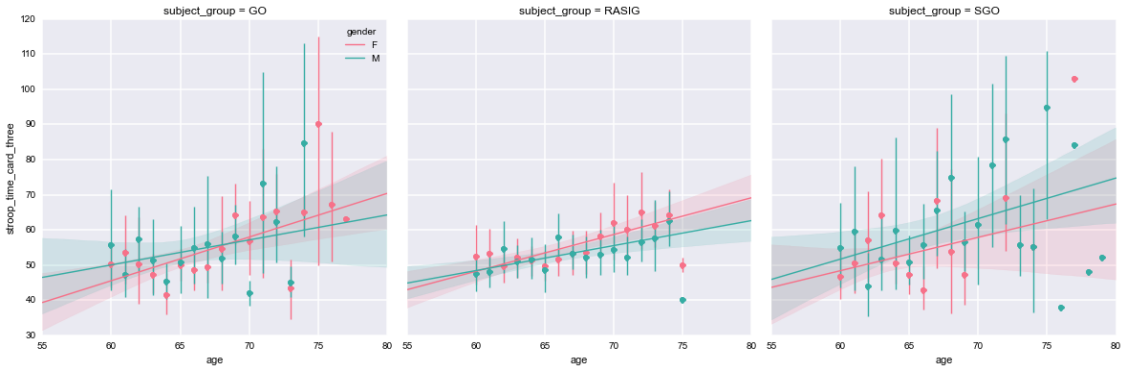


Fig. 31 Stroop Test Card 3and age linear regression

4.2.8.4 Digit Symbol Substitution Task (DSST)

The processing speed of volunteers was assessed with the Digit-Symbol Substitution Task (Lezak et al., 2004). The number of correct digit-symbol combinations within 90 seconds was used for analysis.

Table 46 Stroop Test Card 3 divided for groups and gender divided for groups and gender

SUBJECT GROUP	GENDER	COUNT	MEAN	STD	MIN	25%	50%	75%	MAX
GO	**M**	176	42.803977	12.317812	13	35	42	51	77
	F	233	44.592275	11.712480	12	37	44	52	75
SGO	**M**	129	40.565891	11.239645	12	33	40	48	71
	F	95	42.957895	10.370443	18	36	42	50.5	69
RASIG	**M**	430	42.677907	11.557058	15	34	43	51	76
	F	440	43.297727	11.557866	10	35	44	51	75

The number of symbols properly replaced lied between 40.5 (SGO) and 44.5 (GO) (Table 46). Even though there were 4 points of difference between the lowest score and the highest no significant difference was noted between groups or gender depending (Table 47). The only element able to have significant influence was age (Table 47) (Fig. 32)

Table 47 Digit Symbol Substitution Task (DSST) and age linear regression corrected for groups and gender

	coef	pvalues	[95\% Conf.	Int.]
Intercept	91.467880	3.84e-84	82.243993	100.691767
Subject_Group[T.RASIG]	-0.041698	0.952	-1.400812	1.317416
Subject_Group[T.SGO]	-2.330457	0.0154	-4.214940	-0.445974
Gender[T.M]	-1.048071	0.0792	-2.218312	0.122170
Age	-0.717171	**3.66e-24**	-0.855789	-0.578553

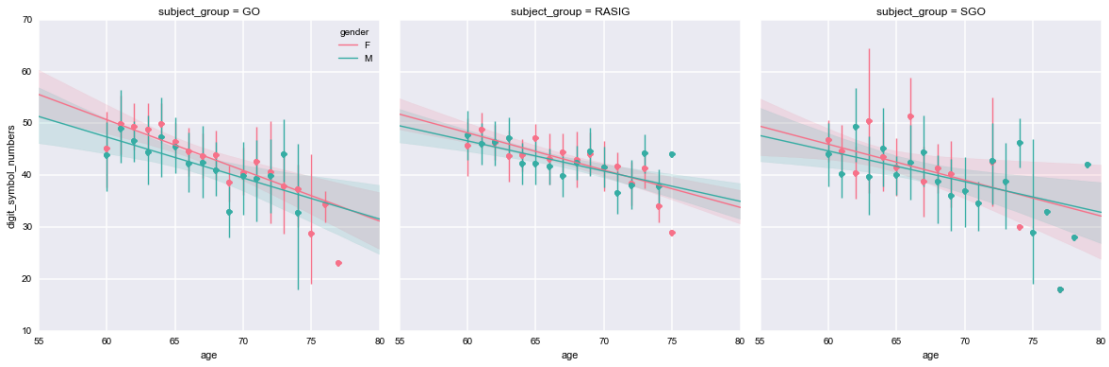


Fig. 32 Digit Symbol Substitution Task and age linear regression

4.3 BIOCHEMICAL PREDICTORS

The main goal of MARK-AGE Project was to identify a set of ageing biomarkers able to measure the biological ageing (or Bioage) along with the ageing rate, discriminating the bioage from the chronological one.

In order to achieve this ambitious target a long list of parameters were investigated (Table 2 page 20):

- **"CLASSICAL"** parameter, for which data from several smaller studies have been published
- **"NEW/SPECIALISED"** parameters, based on recent preliminary data
- **"NOVEL"** parameters, based on recent research on mechanistic aspects of ageing

MARK-AGE project generated a tremendous amount of data from which bioinformatics extracted a robust set of biomarkers, biochemical and phenotypical regressors (not shown in this dissertation). In other words the data were analyzed in order to find relevant biochemical and phenotypical signatures that allowed to evaluate the bioage of an individual, to be confronted with the anagraphical one. This dissertation would propose a first approach for a simple model of "bioage"

First of all, the **BEST BIOCHEMICAL REGRESSORS/PARAMETERS** (Fig. 33) were ranked in Female and Male of GO-SGO-RASIG volunteers.

FEMALE			MALE		
1	0.758	ELOVL_2_CpG_9-17_pca	1	0.747	ELOVL_2_CpG_9-17_pca
2	0.624	S_p6_n_glycan	2	0.55	dehydroepiandrosteron sulfate [$\mu g/dl$]
3	0.618	ELOVL_2_CpG_18-24_pca	3	0.545	ELOVL_2_CpG_18-24_pca
4	0.606	S_log_p1_p6	4	0.451	FHL2_CpG_9-20_pca
5	0.489	S_p2_n_glycan	5	0.397	ELOVL_2_CpG_2-8_pca
6	0.482	dehydroepiandrosteron sulfate [$\mu g/dl$]	6	0.346	Plasma Lycopene [$\mu mol/l$]
7	0.473	S_p1_n_glycan	7	0.287	ELOVL_2_CpG_28.29
8	0.466	FHL2_CpG_9-20_pca	8	0.286	alpha2 macroglobulin [mg/dl]
9	0.464	ELOVL_2_CpG_2-8_pca	9	0.285	S_p6_n_glycan
10	0.458	Ferritin [ng/ml]	10	0.272	Prostate specific antigen [ng/ml]
11	0.359	Fibrinogen [mg/ml]	11	0.266	S_log_p1_p6
12	0.338	Urea [$mmol/l$]	12	0.259	ELOVL_2_CpG_27
13	0.316	Serum glucose [$mmol/l$]	13	0.256	Fibrinogen [mg/ml]
14	0.312	Plasma Alpha.tocopherole [$\mu mol/l$]	14	0.256	Albumin [g/l]
15	0.305	Threonine/Lactate 10^{-6}	15	0.251	Serum glucose [$mmol/l$]
16	0.295	Triglycerides [$mmol/l$]	16	0.247	Creatinine urine 10^{-6}
17	0.287	VLDL2 Triglycerides [mg/dL]	17	0.236	S_p2_n_glycan
18	0.286	Uric acid [mg/ml]	18	0.224	Urinary 8-isoprostane (direct)
19	0.281	Plasma Cysteine [$\mu mol/l$]	19	0.223	CD16+CD56+/CD45 +
20	0.275	VLDL2 Cholesterol [mg/dL]	20	0.223	Tetanus IgG antibodies

Fig. 33 List of best biochemical regressors sorted by descending Spearman's rank correlation coefficient

The most age correlated parameter was ELOVL2- CpG9-17-pca in both genders. ELOVL2 is a fatty acid elongation enzyme and its methylation status show a high correlation with chronological age.

Extremely remarkable is the methylation status of specific CpG of ELOVL2 promotor : CpG9-17; CpG19-24; CpG2-8 belonging to the same island and relevant for both males and females.

Unlike ELOVL2 the other regressors tend to be more gender specific. In females, top-seven positions are mainly filled with N-glycans-specific molecules along with DHEA - Dehydroepiandrosterone sulfate (more significant in males) while in men the methylation status of CpG islands ELOVL2 and FHL2, another gene with methylation status found to be strongly associated with chronological age (Garagnani et al., 2012) are the protagonist along with DHEA and Lycopene plasma level.

With the identification of the best biochemical and phenotypical biomarkers, it was possible to calculate the "predicted age" by a linear model fitting the best correlations (lines red in figure 34 and 35) thus able to "detect" the "Bio-age". Analyzing the graphical distribution of "Bio-age" in GO and RASIG volunteers (separating males from females) the discrepancy between the two groups was immediately evident. As a matter of fact GO subjects (both males and females) apparently seemed to be younger than RASIG, being most of the data shifted below the red line in the younger area.

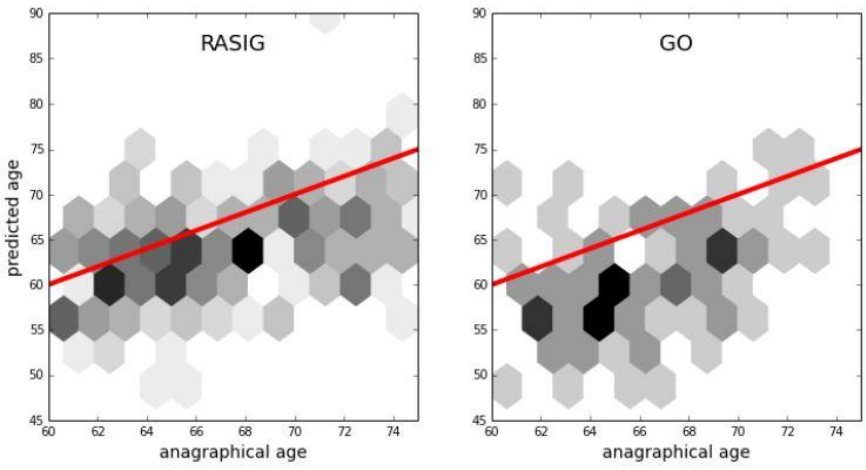


Fig. 34 GO and RASIG males "Bio-age"

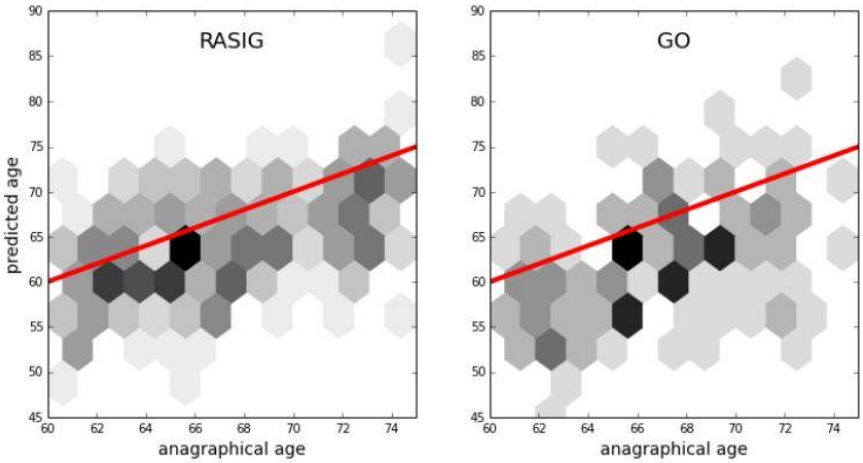


Fig. 35 GO and RASIG females "Bio-age"

4.4 HEALTH, FUNCTIONAL AND COGNITIVE STATUS CORRELATIONS

The possible relationship between, biomarkers and health status of the elderly in analysis has been examined.

4.4.1 ELOVL2 and FHL2 metilation status

Relation between the number of pathologies and ELOVL2 and FHL2 metilation status:

The possible correlation between the number of pathologies affecting volunteers and the metilation status of ELOVL2 and FHL2 was tested (Table 48 and 49). Unfortunately no significant correlation was found in both cases (Fig. 36-37).

Table 48 Number of pathologies and ELOVL_2_CpG_9_17_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-2.154610	9.69e-10	-2.845270	-1.463951
Subject_Group[T.RASIG]	**0.603929**	**7.17e-09**	0.399387	0.808471
Subject_Group[T.SGO]	-0.026493	0.86	-0.320978	0.267992
Gender[T.M]	-0.632632	**1.32e-17**	-0.777784	-0.487479
ELOVL_2_CpG_9_17_pca	0.313394	0.717	-1.383616	2.010403
Age	0.077429	**2.01e-53**	0.067566	0.087292

Table 49 Number of pathologies and FHL2_CpG_9_20_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	-2.187363	2.16e-12	-2.797757	-1.576969
Subject_Group[T.RASIG]	0.655841	**6.94e-09**	0.433922	0.877760
Subject_Group[T.SGO]	-0.037673	0.811	-0.346791	0.271445
Gender[T.M]	-0.587358	**1.51e-13**	-0.743211	-0.431504
FHL2_CpG_9_20_pca	-0.681305	0.389	-2.232868	0.870259
Age	0.084472	**1.39e-77**	0.075593	0.093352

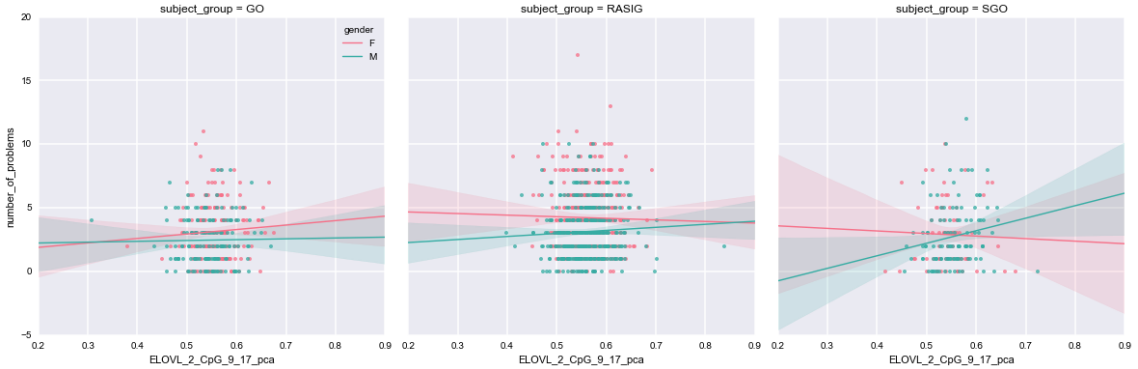


Fig. 36 Number of pathologies and ELOVL2 metilation status logistic regression

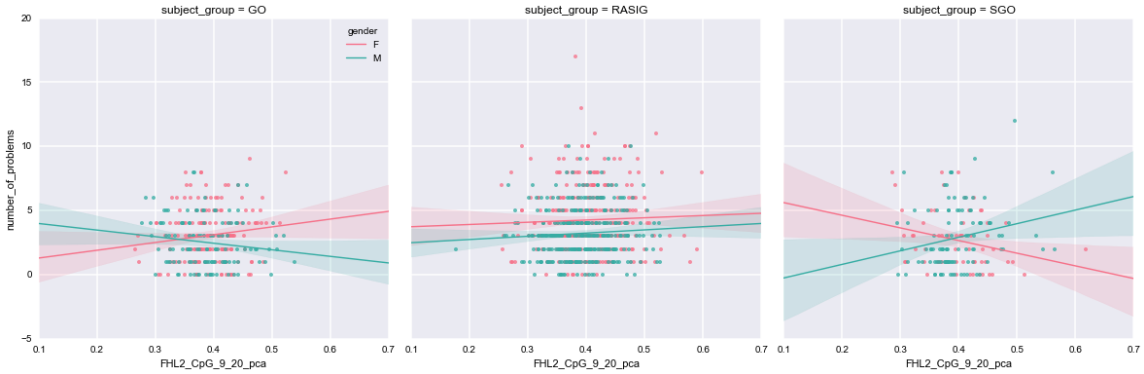


Fig.37 Number of pathologies and FHL2 metilation status logistic regression

Relation between Chair Stand Test and ELOVL2 and FHL2 metilation status:

Chair Stand Test is a simple measure of lower extremity strength and a great indicator of disability. It is frequently used for example to examine functional status (Hammaren & Lindberg 2005, Suzuki et al. 2001, Liikavainio et al. 2008), lower extremity muscle

force/strength (Newcomer et al. 1993, Brown et al. 1995, Hughes et al. 2004, Manini et al. 2005, Bohannon et al. 2005), or to discriminate between fallers and non-fallers (Campbell et al. 1989) in elderly population.

Analysis showed that no significant correlation was found in both cases (Table 50 and Table 51)

Table 50 Chair Stand Test and ELOVL_2_CpG_9_17_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	5.520162	7.18e-06	3.109533	7.930791
Subject_Group[T.RASIG]	-0.936942	**1.3e-08**	-1.259904	-0.613979
Subject_Group[T.SGO]	0.039848	0.863	-0.414104	0.493800
Gender[T.M]	-0.520529	**0.000202**	-0.795009	-0.246049
ELOVL_2_CpG_9_17_pca	2.196952	0.196	-1.134552	5.528455
Age	0.059129	**0.000868**	0.024328	0.093930

Table 51 Chair Stand Test and FHL2_CpG_9_20_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	5.721174	4.8e-06	3.269295	8.173054
Subject_Group[T.RASIG]	-0.755719	**2.56e-05**	-1.107568	-0.403870
Subject_Group[T.SGO]	0.437997	0.0764	-0.046549	0.922544
Gender[T.M]	-0.508478	0.000922	-0.809252	-0.207704
FHL2_CpG_9_20_pca	2.483022	0.0852	-0.344138	5.310182
Age	0.057244	0.002	0.020930	0.093557

Relation between Handgrip strenght and ELOVL2 and FHL2 metilation status:

Muscle strength is supposed to be a potential predictor of declining health in elderly. It was demonstrated that poor handgrip strength can predict not only ADL dependency but also co cognitive decline (Taekema et al. 2010).

No significant correlation was found (Table 52-53)

Table 52 Handgrip strength and ELOVL_2_CpG_9_17_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	59.305658	6.83e-84	53.316001	65.295314
Subject_Group[T.RASIG]	-1.857790	**7.32e-06**	-2.669787	-1.045793
Subject_Group[T.SGO]	-0.395082	0.499	-1.539616	0.749452
Gender[T.M]	15.528736	0	14.840862	16.216611
ELOVL_2_CpG_9_17_pca	-8.274281	0.0501	-16.551347	0.002784
Age	-0.404182	**3.72e-20**	-0.490328	-0.318035

Table 53 Handgrip strength and FHL2_CpG_9_20_pca logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	55.888003	1.06e-70	49.726215	62.049790
Subject_Group[T.RASIG]	-2.229399	**1.14e-06**	-3.127400	-1.331398
Subject_Group[T.SGO]	-1.064279	0.093	-2.306217	0.177658
Gender[T.M]	15.385560	0	14.621749	16.149371
FHL2_CpG_9_20_pca	3.348769	0.361	-3.831453	10.528991
Age	-0.437974	**6.05e-21**	-0.529401	-0.346547

Correlations with cognitive status was also tested but even in this case no significant correlations were found

4.4.2 N-Glycans

Health status of MARK-AGE volunteers was correlated also with N-Glycans. Possible correlations with peak 1, peak 6 and the log of p1/p6 ratio of N-Glycans were tested. Unfortunately the performed analysis showed no significant correlations.

4.4.3 Serum glucose level and serum glycosylated haemoglobin level

The connection between diabetes and cognitive impairment has been established but the influence of glucose regulation on attentional functions, which are sensitive to aging, is still unclear. For this reason the possible correlation between the cognitive tests analysed in this dissertation and serum glucose level along with serum glycosylated haemoglobin level was tested.

Relation between SMMSE and glucose and Glycosylated haemoglobin serum level:

No significant correlation was found (table 54 and Table 55)

Table 54 SMMSE and serum glucose level logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	94.380518	5.06e-77	84.422167	104.338868
Subject_Group[T.RASIG]	0.064707	0.928	-1.348205	1.477619
Subject_Group[T.SGO]	-1.989162	0.0464	-3.946741	-0.031584
Gender[T.M]	-0.998573	0.112	-2.231587	0.234440
Serum glucose	-0.458059	0.0577	-0.931070	0.014951
Age	-0.724109	**2.27e-22**	-0.869983	-0.578235

Table 55 SMMSE and serum glycosylated haemoglobin level logistic relation corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	98.320922	5.56e-70	87.423836	109.218008
Subject_Group[T.RASIG]	0.373057	0.599	-1.017926	1.764039
Subject_Group[T.SGO]	-2.058795	0.0376	-3.999789	-0.117802
Gender[T.M]	-1.122736	0.0681	-2.328892	0.083421
Glycosylated haemoglobin	-1.003662	0.0301	-1.910872	-0.096453
Age	-0.728885	**2.24e-23**	-0.872292	-0.585478

Relation between Stroop Colour Word Test and glucose and Glycosylated haemoglobin serum level:

A very significant correlation was found between Stroop Colour Word Test Card 1 and serum glucose level (Table 56)

Table 56 Stroop Test Card 1 and serum glucose level logistic regression corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	13.648532	1.27e-11	9.698654	17.598409
Subject_Group[T.RASIG]	-1.544746	**9.3e-08**	-2.111731	-0.977761
Subject_Group[T.SGO]	0.114048	0.777	-0.674186	0.902281
Gender[T.M]	-0.202750	0.419	-0.694730	0.289231
Serum glucose	-0.385485	**5.51e-05**	-0.572835	-0.198136
Age	0.141383	**1.84e-06**	0.083293	0.199474

No significant correlation was found with serum glycosylated haemoglobin level.

Stroop Test Card 2 had showed no significant difference with glucose and glycosylated haemoglobin.

Stroop Test Card 3 showed a significant correlation with serum glucose level (Table 57)

Table 57 Stroop Test Card 3 and serum glucose level logistic relation corrected for groups, gender and age

	coef	pvalues	[95\% Conf.	Int.]
Intercept	3.860489	0.568	-9.379903	17.100882
Subject_Group[T.RASIG]	1.046222	0.282	-0.859303	2.951746
Subject_Group[T.SGO]	2.417639	0.0743	-0.237641	5.072919
Gender[T.M]	0.395860	0.639	-1.256190	2.047911
Serum glucose	-1.199925	**0.000179**	-1.827680	-0.572170
Age	0.798456	**9.36e-16**	0.603688	0.993224

No correlation was found with serum glycosylated haemoglobin level.

No correlation between Digit Symbol Substitution Task (DSST) e 15 Picture Learning Test with glucose and glycosylated haemoglobin serum level was found.

5. DISCUSSION

5.1 MARK-AGE RECRUITMENT

The main goal of the Large-Scale Integrated Project MARK-AGE was to recruit a large group of probands from different European countries supposed to be characterized by different rates of ageing to identify a robust set of ageing biomarkers across a range of physiological systems.

MARK-AGE recruitment has been successful: 2336 RASIG, 566 GO, 323 SGO, 53 Down-Syndrome 59 Cockayne-Syndrome and 50 Werner-Syndrome patients were recruited. In order to confirm the biological measurement robustness, 100 donors selected from the whole study population has been re-sampled within 3-6. Moreover, a small longitudinal study was performed by re-testing about the 12% of volunteers after 3 years from the first recruitment

The success of MARK-AGE Project lies not only in achieving the number of programmed volunteers but thanks to the strategy behind the recruitment we may be able to analyse age-gender effects, identify early ageing biomarkers and investigate a wide range of environmental, geographical and cultural effects on population. In fact MARK-AGE recruitment design has been carefully thought and formulated keeping in mind three key points: gender, age and geography.

a. **GENDER**: males and females volunteers were recruited close to 50% in each age class. According to literature mortality rate is characterized by a gender effect, as a matter of fact women live longer than men albeit affected by a higher frequency of disabilities and comorbidities (Oksuzyan et al., 2008). The explanation behind this apparent contradiction has not been clarified yet but recent findings on age-dependent methylation pattern of sexual chromosomes (Capri et al., 2013; Gentilini et al., 2012; 2013) seemed to be different suggesting a possible role.

b. **AGE:** MARK-AGE has focused its attention on a specific range of age 34 and 75 years in order to detect early ageing biomarkers. This choice has been made taking into account that the human beings, during their lifetime, goes through many phases of life and the age range between 60 and 75 years appears to be a critical temporal

window (Rauser et al., 2006) and likely the most interesting to identify biomarkers able to discriminate successful and unsuccessful ageing.

c. **GEOGRAPHY:** according to literature geographical or environmental factors could have an influence on several biological parameters such as methylation patterns (Pirazzini et al., 2012). The analysis carried out on volunteers recruited by 11 different European beneficiaries, distributed from North to South could help to better define possible geographical impact.

As mentioned above MARK-AGE Project has been a success even though extremely challenging as well as the majority of population-based studies involving older adults. In fact recruiting volunteers from this age group is particularly demanding and require a long and accurate planning that begins long before the first contact with potential candidates (Bonk 2010). The identification of sample size and power requirement, targeting of participants, creation of protocols, localization of facilities and so on require a careful analysis and a high level of organization that is able to ensure that the recruitment can proceed without interruptions or problems. And this is what happened with MARK-AGE Project. In fact the success of the study is partially due to the almost perfect organizational process that every recruitment centres has been able to set up. However, it is fair to add, that great part of MARK-AGE success is due to recruiters commitment as the difficulties to be overcome, sometimes unexpected, were many.

It is important to emphasize that fundamental was the great enthusiasm of contacted volunteers (according to UNIBO experience). Volunteers showed great desire to be part of such ambitious project and this not for personal gain but with the intent to improve the health of future generations.

5.2 BIOCHEMICAL AGE PREDICTORS AND THE "BIOLOGICAL AGE"

The strong correlation between the methylation status of in ELOVL2 and FHL2 CpG islands and age is surely one of the most important finding in MARK-AGE Project and it was a confirm of previously results (Garagnani et al., 2012).

ELOVL2 (ELOVL fatty acid elongase 2) belongs to ELOVL gene family (elongation-of-very-long-chain-fatty acids) that encode elongases (Jakobsson et al., 2006) and encodes for a transmembrane protein, mainly expressed in the liver and involved in the synthesis of ώ3 and ώ6 polyunsaturated fatty acids (PUFA) (Leonard et al., 2002, Garagnani et al., 2012). Polyunsaturated fatty acids (PUFA) play an important role in many physiological processes such as modulation of inflammation, energy production and maintenance of cellular membrane integrity and their high plasma concentrations have been shown to have beneficial effects on cardiovascular disease and mortality (Tanaka et al., 2009).

FHL2 (four and a half LIM domains 2) gene is located on chromosome 2q12–q14 (Johannessen et al., 2006) and belongs to the FHL family (Genini et al., 1997). It is expressed mainly in heart, a little lower in ovary, a marginally in brain, liver and lung (Tanahashi and Tabira, 2000, Fung Ng et al., 2011) and it is also known to be down-regulated in rhabdomyosarcoma LIM (DRAL) domain protein (Fung Ng et al., 2011)

Garagnani et al. in 2012, studing on a cohort of 500 subjects of different ages, strongly and unequivocally correlated the methylation level of three regions, the CpG islands of ELOVL2, FHL2, and PENK genes (all located in the respective gene promoter) with age.

For this reasons ELOVL2 and FHL2 genes, that showed the most significant results in Garagnani study, were chosen to be investigated in MARKAGE. N-Glycans (in particular peak 1, 2, 6 and log p1/6), along with ELOVL2 and FHL2, have been identified as one of the best biomarkers. Protein glycosylation is the enzymatic addition of oligosaccharides (also known as glycans) to proteins and represents one of the most frequently form of co-translational modification of proteins (Apweiler et al., 1999). Unlike what normally happens to RNA and proteins, glycans are being synthesized without a genetic template that pre-

determines their final structure and they are the results of complex interaction between hundreds of different genes (Pucic et al., 2010). N-Glycans action takes place on many fronts, in fact they are involved in the folding and conformational stability of many proteins, mediate host–pathogen interactions and aspects of innate immunity etc. (Vanhooren et al., 2009). Serum N-glycan profiling has been indicated to have a potential to predict prognosis in patients undergoing haemodialysis and serum N-glycans alterations has been associated also with renal cell carcinoma (Hatakeyama et al., 2013). According to latest studies N-glycans can be a predictive prognosis factor in ulcerative colitis (Miyahara et al., 2013).

N-glycans association with age was proved for the first time in 2009 by Vanhooren et., al showing that two N-glycan structures (NGA2F and NA2F) present in human blood glycoproteins changed with ageing. The following year it was showed that the log of the ratio of two glycans (NGA2F and NA2F), called GlycoAgeTest, remained steady up to the age of 40 years and thereafter gradually increased to reach its highest level in nonagenarians (Vanhooren et., 2010). Recent data suggested that the N-glycomic shift observed in aging could be related not only to inflammation but also to alteration of important metabolic pathways (Dall'Olio et al., 2013)

With all these premises N-Glycans and particularly NGA2F and NA2F resulted to be one of the best age predictors.

The identification of this novel ageing biomarkers open a brand new and wide range of knowledge and it can be stated that now we can have access to a new level of comprehension of ageing mechanisms.

Using these biomarkers it has been possible to formulate a model able to discriminate between bio-age and chronological age and this can be considered a tremendous result. The main assumptions of MARK-AGE namely that exist different ageing rate and that Geha Offsprings are biologically younger than age-matched normal population was confirmed.

5.3 GO, SGO AND RASIG HEALTH, FUNCTIONAL AND COGNITIVE STATUS AND THEIR RELATION WITH AGEING BIOMARKERS

The main goal of this dissertation was to outline the health status of MARK-AGE elderly volunteers and eventually identify the presence of different ageing trajectories between the three group of recruited volunteers presumed to be characterized by distinctive ageing rate (GO, SGO and RASIG).

Before starting the discussion it is important to underline that the sample in analysis is surely a "selected sample" since probably the subjects with the worst health status are to be found among those who refused to participate to the project.

The aging process is frequently characterized by the presence of multimorbidity, disability, and frailty (Christensen et al. 2009). Finding elderly individual who presents only one single disease impairing the functionality of a single apparatus is really a rare occurrence (Nobili et al. 2011, Marengoni et al. 2008, Abete 2004). In order to get an idea of the magnitude of the phenomenon is sufficient to say that the multimorbidity affects more than half of the elderly population (Marengoni et al. 2011). Furthermore the presence of multimorbidity increases with age and worsens the indices of the perceived quality of life. Our sample accurately reflects the world situation, in fact MARK-AGE volunteers are characterized by multimorbidity and it is noteworthy that more than one-fifth of the sample reported to have other pathologies in addition to those investigated by the project. Moreover regarding multimorbidity and previous morbidity, the collected data may be underestimated since the medical history was reported without the support of medical reports but only confirmed by the ongoing pharmacological therapy.

The diseases with higher prevalence among MARK-AGE participants were vision and hearing impairment, hypertension, hypercholesterolemia and arthritis that, as expected, affected mostly females. The percentages of subjects with vision deficits are extremely high. It is important to remember that the sensorial disabilities, such as impaired vision and hearing, may affect the perceived quality of life and elderly level of social integration as it jeopardizes their ability to communicate, leading to a limitation of normal social relationships. Moreover the vision and hearing impairment are important risk factors for falls and fractures and affect

the ability of the elderly to perform normal activities of daily living, which ultimately leads to growing needs of social and health care.

Extremely diffused is the use of medications with remarkable percentages in all three recruited groups. Multiple medication use (prescription or OTC) is a common concern in relation to seniors' health since elderly people are prone to drug-related problems such as inappropriate prescribing, adverse drug reactions, non-compliance with prescribed medications etc. (Elmståhl and Linder 2013)

As reported in literature, females volunteers showed to have a worst health status.

The sample in analysis showed no disability and this could be due to the phenomenon of self-selection we mentioned before. However a significant presence of incontinence was found in women, as reported in literature. In fact urinary incontinence is a common geriatric syndrome that affects at least 1 in 3 older women (Goode et al., 2010). Incontinence problems have been found to be associated with higher incidence of many other health problems such as obesity and diabetes but this occurrence has not been evaluated in this dissertation.

The assessment of functional performance was carried out with Chair Stand Test and measurement of handgrip strength and both showed deterioration with age, as reported in literature.

Regarding cognitive status no subject showed cognitive impairment in MMSE evaluation but probably, as previously said, the more compromised individuals refused to participate. Mnemonic properties along with responsiveness to various stimuli tend to slowdown with age in an almost physiological phenomenon. All the performed cognitive test showed a worsening of scores as age increases. In 15 Pictures Learning Test females performed better than males but no reference of this was found in literature. Particularly interesting was the relation found between the execution of Stroop Test Card 1 and 3 and serum glucose level. Even though diabetes has been associated with cognitive decline and dementia, the relationship between the degree of hyperglycemia and cognitive status still remains unclear (Cukierman-Yaffe T, et al., 2009). Although data in the literature on this subject are still few, it has been suggested that glucose regulation may transiently influence performances of metabolically healthy older

adults on tasks requiring switching attention (Gagnon et al., 2011). Surely this point is definitely worth to be further investigated.
Analysing the obtained data, two aspects are immediately evident:

1. No significant difference between GO and SGO volunteers was found.

2. RASIG volunteers showed to have an objectively worst health status confirming the initial hypothesis according to which individuals can have different ageing trajectories and that chronological age and biological age are not specular.

Regarding the absence of differences between GO and SGO it is necessary to say that this may be due, at least in part, to the fact that the number of recruited SGO was much lower than the planned and this may have contributed to flatten the possible differences between the two groups.

Concerning RASIG health status, as anticipated, all data indicates that RASIG volunteers are characterized by a worse health status. Proof of this is the fact that RASIG are affected by a significantly higher number of pathologies and use a significant higher number of medications. They showed a higher prevalence of vision impairment and previous myocardial infarction and tend to be more affected by cardiovascular diseases. Considering all the risk factors that can negatively influence the evolution of cardiovascular disease such as hypertension, diabetes and hypercholesterolemia, RASIG volunteers tend to be more metabolically unbalanced. Considering the functional status and in particular handgrip strength measurement they showed to perform significantly worse than GO and SGO while in Chair Stand Test they obtained the best results. No significant differences were found in cognition.

We can therefore conclude that considering all these aspects, GO and SGO volunteers definitely have a better health status than RASIG.

One goal of this dissertation was to explore possible relations between health status and some of the best biochemical regressors, in particular the metilation status of ELOVL2 and FHL2 CpG island along with N-Glycans. Contrary to all expectations no significant correlations was

found. At the moment we are not able yet to explain the reason for this “negative” result. Considering the huge amount of data generated by MARK-AGE project it is possible that the real motivation lies in the fact that the most appropriate variables were not selected and the number of parameters with which we can investigate possible supplementary correlations are still many. Without doubt this event further stimulates our inquisitiveness and in a sense encourages us to think “outside the box” in order to find answers.

5.4 CONCLUSIONS AND POSSIBLE FUTURE DEVELOPMENTS

The results achieved by MARK-AGE Project have been absolutely remarkable and we think that this is the beginning. In this moment only small portions of knowledge has been revealed and definitely further analysis allow us to achieve a large part, if not all of the MARK-AGE prefixed goals. We hope that identification of ageing biomarkers along with Bio-age can be effective in the detection of individuals at high risk of developing age-associated diseases or disabilities and therefore a useful prevention tool in order to program prophylactic interventions or early-stage treatments of age-related diseases.

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