Executive Summary:
The rate of ageing in humans is not uniform, due to genetic heterogeneity and the influence of environmental factors. Age-related changes in body function or composition that could serve as a measure of “biological” age and predict the onset of age-related diseases and/or residual lifetime are termed “biomarkers of ageing”. Many candidate biomarkers have been proposed but in all cases their variability in cross-sectional studies is considerable, and therefore no single measurement has so far proven to yield a useful biomarker of ageing on its own, probably due to the multi-causal and multi-system nature of ageing. We propose to conduct a population study (3,700 probands) to identify a set of biomarkers of ageing which, as a combination of parameters with appropriate weighting, would measure biological age better than any marker in isolation. Two large groups of subjects will be recruited, i.e. (1) randomly recruited age-stratified individuals from the general population covering the age range 35-74 years and (2) subjects born from a long-living parent belonging to a family with long living sibling(s) already recruited in the framework of the GEHA project. For genetic reasons such individuals (“GEHA offspring”) are expected to age at a slower rate. They will be recruited together with their spouses as controls, thus allowing initial
validation of the biomarkers identified. (3) A small number of patients with progeroid syndromes will also be included in the study. A wide range of candidate biomarkers will be tested, including (a) “classical” ones for which data from several smaller studies have been published; (b) “new” ones, based on recent preliminary data, as well as (c) “novel” ones, based on recent research on mechanistic aspects of ageing, conducted by project participants. Bioinformatics will be used in order to extract a robust set of biomarkers of human ageing from the large amounts of data to be generated and to derive a model for healthy ageing.

Project Context and Objectives:
In MARK-AGE study two large groups of subjects were recruited. The first comprised of 2,320 randomly recruited age-stratified individuals from the general population (RASIG) in total from several different geographical regions of Europe. Equal numbers of men and women have been enrolled, comprising the same numbers of individuals 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. This group will be representative of the “average population ageing rate”.

The second group comprised of subjects born from a long-living parent belonging to a family with long-living sibling(s), such as the 90+ sibpairs recruited within the framework of the EU Integrated Project GEHA, and henceforth designated GEHA offspring (GO) (700 subjects). GO cover the age range of 55-74 years. According to data from the recent literature, indicating that offspring of long-living parents age in a “better” way than controls born from non long-living parents, GO are predicted to age at a slower rate than the average population.

Within the MARK-AGE project, the GO subjects will therefore be compared with their spouses, henceforth designated spouses of GEHA offspring (SGO) (about 400 subjects). Systematic comparison of GO and SGO cohorts will provide a unique opportunity for a first validation of the biomarkers identified in the cross-sectional study of the RASIG subjects. It is expected that GO will display a lower biological age than SGO. The project also takes advantage of the fact that some relatively rare ‘segmental progeroid syndromes’ present characteristics of dramatically accelerated ageing and premature death from typical ageing-associated diseases. This is the case for subjects affected by Down’s syndrome (DS), Werner’s syndrome (WS) or Cockayne’s syndrome (CS).

From all subjects enrolled, anthropometric, clinical and social data have been collected in a standardised fashion. Upon written informed consent, the following set of information was obtained by using a standardised questionnaire: Demographic information: family composition, marital status, education, occupation, and housing conditions. Lifestyle: use of tobacco and alcohol, daily activities. Functional status: Activities of Daily Living (ADL) and Norton Scale Cognitive status: STROOP test, 15-picture learning test. Health status: present and past diseases, self-perceived health, number and type of prescribed drugs. Mood: ZUNG depression scale.

A physical examination of all subjects comprised measurement of the following “classical” candidate biomarkers: Body mass index, waist and hip circumference, blood pressure at rest, heart rate at rest, lung function, five-times chair standing and handgrip strength.

All subjects were asked to donate blood (55 ml) by phlebotomy after overnight fasting. The blood sample was processed to obtain plasma, serum, peripheral blood mononuclear cells (PBMC). PBMC were cryopreserved and all the other components will be frozen down. Buccal mucosal cells were also collected (using a kit) as well as spot urine samples.

DNA-based markers: Our overarching hypothesis is that the presence of proficient systems to prevent/repair damage and mutation to the nuclear genome, including telomere shortening, and to the
mitochondrial genome should help retard the ageing process in many if not all tissues. Therefore these cellular functions have a potential to serve a biomarkers of ageing. The following research tasks have specifically been addressed:

- We have studied the maintenance of the epigenome by analysing gene expression patterns in PBMC and cytosine methylation status. DNA methylation will be correlated with the possible age-dependent expression level of some genes whose expression has been associated with ageing or longevity.
- The ageing-dependent on decline of DNA repair was evaluated by functional analysis of the repair of DNA strand breaks induced by X-rays and in studies on cellular poly(ADP-ribosyl)ation capacity and PARP-1 expression levels.
- Attention was also directed towards telomere length, which is being correlated with modifications of subtelomeric DNA methylation pattern.
- The question of an age-related accumulation of mutations in mtDNA will be addressed by quantifying the level of heteroplasmy. Special attention will be paid to heteroplasmy of the mtDNA control region.
- Donors are being stratified for their APOE genotype to correlate this with the type of ageing, i.e. successful or unsuccessful ageing.

Markers based on proteins and their modifications: Protein maintenance systems, viewed as potential biomarkers of ageing, are being monitored at the levels of enzymatic activity, protein expression, and RNA expression. The following research tasks will specifically be addressed:

- Analysis of the N-glycomic changes in glycoproteins from blood of all donors. Urine glycoprotein changes are being studied in a subset of subjects.
- ApoJ/CLU levels in serum from all donors
- AGEs in plasma by fluorescence spectroscopy and by immunological analysis of carboxy-methyllysine, pentosidine, arg-pyrimidine and imidazolone
- Protein damage in blood at different levels, i.e. activities of proteasome and methionine sulfoxide reductases in PBMC lysates; RNA levels of representative proteasome subunits (catalytic and regulatory) and methionine sulfoxide reductases A and B; and protein levels of representative proteasome subunits and methionine sulfoxide reductases A and B in PBMC lysates

Immunological markers: Thymic output is known to decline with age. A robust immunological memory is known to be a guarantor of health in adults and in particular in elderly persons, while chronic latent infections such as with CMV have been shown to be associated with shorter life expectancy. In view of previous results we suggest that during senescence chronic antigenic load as well as oxidative stress cause decreased lymphocyte susceptibility to Damage-Induced Cell Death (DICD) and, on the other hand, increased susceptibility to Activation-Induced Cell Death (AICD). As an intact equilibrium between survival and elimination of immune cells may be decisive for intact immune function and health we are studying DICD and AICD in T cells from donor samples. The following research tasks are specifically addressed:

- Analysis of total IgG, IgE, IgM and IgA, serum/plasma concentrations of 14 cytokines, blood counts and differential blood counts (performed by the recruiters locally), and phenotyping of T cells, B cells NK cells and monocytes by immunofluorescence in proband samples
- Analysis of the number of sjTRECs; it is of particular interest to analyse whether sjTREC concentrations differ in persons with and without latent viral infections such as CMV, HHV-6 and HHV-7
- Analysis of antibodies and cellular immunity (IFN gamma production by Elispot) against measles and mumps virus (typically childhood exposure) in order to assess long-term immunological memory
• Analysis of antibodies and cellular immunity to highly conserved proteins of the influenza virus (NP and M proteins) as well as tetanus (an agent against which most adult persons are vaccinated at regular intervals) in order to assess short-term immunological memory
• Analysis of immune responses against CMV, in order to assess the effect of latent viral infection
• Analysis of autoantibodies against thyroglobulin (as an example of a tissue-specific antigen) and antinuclear antibodies (as example for a systemic immune response)
• Analysis of susceptibility to Damage-Induced Cell Death (DICD) and Activation-Induced Cell Death (AICD), respectively, by using apoptosis markers.

Clinical chemistry, hormones and markers of metabolism: In the literature a plethora of classical clinical chemistry parameters have been proposed as potential biomarkers of ageing. Prominent examples are parameters of carbohydrate and lipid metabolism or hormones. We have selected the most promising ones for inclusion in the MARK-AGE project and we have added several new potential biomarkers related with metabolism that have emerged in the recent work of some Beneficiaries.

Oxidative stress markers: The purpose is to analyse a set of parameters of oxidative stress parameters, vitamins and trace elements in human blood, serum, urine and buccal mucosa cells. Preference is given to new technologies for the assessment of oxidation markers, and to markers already established and suitable for adaptation to high-throughput formats. The following candidate biomarkers are specifically addressed: Malondialdehyde, Carbonylated and nitrated proteins, Establishment of a new, sensitive and amino-acid specific method of the measurement of protein oxidation, and assessment of its power as a biomarker of ageing, Oxidation of LDL, NO metabolic-pathway products NOx, Isoprostanes, Cellular glutathione, Vitamin content (\(\alpha\)-tocopherol, \(\beta\)-carotene and ascorbate) of serum and buccal mucosal cells, Trace elements (Zn, Cu, Se and Fe) in blood / serum.

Emergent biomarkers of ageing from model systems and novel methodological approaches: Whilst conventional biomarkers of disease have been established by hypothesis-driven approaches based on an underlying knowledge of the disease process or serendipity, studies which focus on identification of biomarkers of healthy ageing are constrained in their ability to follow individuals over long periods of time until their chronological age deviates from their biological age. To overcome this, we are adopting parallel, systematic approaches to investigate putative biomarkers in specific ageing cohorts (as defined in WP1) alongside the study of models of accelerated ageing, such as progeroid syndromes (in humans and mice) and induced senescence in leukocytes from subjects of different ages. We are using both established and novel approaches to search for biomarkers of ageing in an iterative process, where markers derived from models will inform in vivo biomarker searches.

Project Results:
Ethical Approval: During the first funding period, one of the top-priority tasks for the Beneficiaries involved in recruitment of MARK-AGE subjects was to obtain ethical approval from the competent authorities in the respective countries. As the ethical requirements differ between countries, it was necessary to make appropriate adaptations. In order to obtain ethical approval, the following documents were created: “Informed consent”, “Participant Information Sheet” and “Synopsis of MARK-AGE project” (see WP1 data annex). These documents were originally created in English and the Beneficiaries involved in the recruitment then translated them into their respective national language, i.e. Dutch, Finnish, French, German, Spanish, Italian and British.
Preparations for Recruitment / Questionnaires: Beneficiaries #01 (UKON) and #21 (UNIBO) took a lead in establishing the questionnaires to be filled out by the MARK-AGE subjects and prepared a draft. The final version was generated in close interaction between all Beneficiaries involved in recruitment and is now perfectly adapted to the needs of the MARK-AGE project. These questionnaires were also first created in English and the recruiting Beneficiaries then translated MARK-AGE 1st progress report 8 them in their respective national language. The English version has been used as template for the MARK-AGE database (see below). Due to the identical layout of the translated versions and the English version, it is now very easy for staff at the various recruitment centres to enter the answers into the database.

Standard Operating Procedures (SOPs): Another very important task was to standardize all the recruitment procedures and thus to provide a set of Standard Operating Procedures (SOPs), which is binding for all MARK-AGE Beneficiaries involved in recruitment. The SOPs cover all aspects of collection, shipment and distribution of biological samples (blood and its components, buccal mucosa cells (BMC) and urine) as well as the anthropometric measurements and questionnaires. Beneficiary #01 (UKON) took a lead in optimizing the laboratory procedures for isolation of peripheral mononuclear blood cells (PBMC) in order to obtain the maximal number of cells from the limited amount of blood (50 ml) to be taken from MARK-AGE subjects. In close cooperation with Beneficiary #19 (UHOH), a very detailed and elaborate master protocol was established for obtaining serum, plasma, whole blood and PBMC as well as for aliquoting of the cells in order to provide all Beneficiaries with the biological material they need for their analyses.

Biobank and Sample Shipment: According to the Technical Annex, the biological samples are to be received by the Biobank under the primary subject code (PSC). At the Biobank samples are to be stored in liquid nitrogen or at -80°C, respectively, re-labelled with the secondary subject code (SSC) and distributed to the Beneficiaries for biochemical or molecular analyses. The Biobank was established by Beneficiary #19 (UHOH); this task included the assignment of dedicated laboratory space; the purchase of heavy equipment (liquid nitrogen tanks and accessories, deep freezers) and central procurement of disposable plastic (tubes, monovettes etc.); and the replacement of the primary subject code (PSC) with the secondary subject code (SSC). The Biobank is also in charge of thawing and splitting the urine, plasma and serum samples of every MARK-AGE subject into several smaller aliquots in order to provide the Beneficiaries with the amounts necessary. By contrast, the cells have to be distributed into small aliquots already at source, i.e. at the recruitment centres, since they are extremely sensitive to freeze-thawing cycles. Beneficiary #19 and Beneficiary #01 also took a lead in setting up a shipment strategy for the samples, including the optimization of the packets size, volume and weight and the elaboration of a shipment plan (recruiters biobank analytical laboratories).

Phenotypic Database: Another important aspect in the first year of MARK-AGE was the creation of the MARK-AGE database as the central site for electronic storage of questionnaire information, anthropometric data and analytical data on blood, urine and buccal mucosal cells from each MARK-AGE subject. Thanks to the close and very intense co-operation between all Beneficiaries involved in recruitment, all possible sources for mistakes and incoherencies associated with electronic data storage, performed via a secure internet link, could be identified and fixed. The procedure of entering data in the database is now very easy for all staff at the various recruitment centres.
database was tested and “rehearsed” repeatedly by the Beneficiaries, in order to prevent any problems during the phase of active recruitment. Another achievement was the establishment of “data views”. The main improvement was the incorporation of an “R” node to the data mining tool KNIME, which expands the possibilities of data analysis and views. The reports generated include histograms, scatter plots and conditional box plots; these are important to understand samples distribution, relationship between biomarkers and their correlation with ageing. The scatter plot displayed a linear regression between biomarker and age of females (red) and males (blue) individuals. Additional information about Subject group (RASIG, GO, SGO), number of individuals analyzed (n), the mathematical formula of the regression line and Pearson’s correlation coefficient was provided. The possibility of a non-normal distribution was also considered by introducing the non-parametric Spearman’s correlation coefficient (rnp) beside the parametric Pearson’s correlation coefficient (rp). In order to get a better view of the data outliers were visually excluded from the plot by cutting 1% of the lower and upper quartiles, respectively. Box plots are used for displaying differences between age groups. An automatic flow creates box plots in ten- or five-year classes. Since the outliers can disturb the viewing of the boxes, an automatic flow was designed to generate two box plots, one showing the original data and a second zooming into the plot until the whiskers have reach the border.

Biological Age Score: The essential achievement was providing a Biological Age Score for women or men, respectively, which is based on a set of 10 different molecular and clinical chemistry biomarkers that have been assessed in blood, blood components or urine. Selection of these parameters and their weighting was done in such a way as to maximize the correlation coefficient between the Biological Age Score and chronological age in the population. Importantly, “GeHA offspring” subjects display a lower Biological Age Score than RASIG subjects of the same chronological age, in perfect agreement with other studies showing that the ageing process in GeHA offspring in the middle age range is slowed down, compared to the general population.

Potential Impact:
The principal impact of this work programme is in the development of a mathematical model to enable calculation of biological age. This will require the concerted activity of European experts in the analysis of existing and yet to be acquired physiological and molecular biomarkers in a unique European ageing population. Importantly, to achieve this outcome the population base will randomly selected of probands between the ages of 35-74 years and will also include a sub-study of progeroid syndrome patients who will be expected to age rapidly. The intellectual property associated with such a model is likely to added economic value to European research. The second impact arising from this project will be the identification of risk markers for accelerated ageing. These will comprise a number of different measures, not previously combined and will allow the identification of individuals at risk of ageing fast / unsuccessfully, through enhanced screening programmes which may be recommended as a consequence of this project.

Insurance industry / Pension schemes: It cannot be ruled out that in the long run, the new knowledge to be generated in the MARK-AGE project could form a basis for redesigning pension schemes and/or life insurance policies, by correcting chronological age (most often the exclusive parameter determining costs or date of payouts) for accelerated / retarded biological ageing. Whilst such knowledge is a potential risk for some individuals, ethical and political aspects of use of the new knowledge on biomarkers will be publicly discussed and ultimately parliaments will reach decisions about the acceptability or any restrictions to be imposed, according to the rules of democracy.
Such knowledge has important implications for development of the third principal impact; development of novel strategies for preventing accelerated ageing. The potential impact of the MARK-AGE project on preventive medicine cannot be overstated. One important aspect of preventative medicine which is being increasingly recognised across Europe is the impact of nutrition and over-nutrition on parameters of ageing. As one of the largest Foods companies in the world, Beneficiary 22 plays a prominent role in representing Industry in areas of policymaking. Work within the current consortium, including the investigation of diet and lifestyle (including physical activity) factors that impact on obesity and insulin resistance, can therefore have a direct and powerful impact on public health initiatives and research funding with potentially wide-reaching effects. The translation of basic research findings to the development of new or existing foods products underlies the basic research strategy of Beneficiary 22. Work planned within the consortium presents several opportunities for successful translation of research findings. Novel insights from collaborations with other partners will lead to potential Foods Category input in such areas as development of diets or new food products with specific components to support healthy ageing.