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MARK-AGE biomarkers of ageing

Alexander Bürkle[a,*], María Moreno-Villanueva[a], Jürgen Bernhard[b], María Blasco[c], Gerben Zondag[d], Jan H.J. Hoeijmakers[e], Olivier Toussaint[f], Beatrix Grubeck-Loebenstein[g], Eugenio Mocchegiani[h], Sebastiano Collino[i], Efstathios S. Gonos[j], Ewa Sikora[k], Daniela Gradinaru[l], Martijn Dollé[m], Michel Salmon[n], Peter Kristensen[o], Helen R. Griffiths[p], Claude Libert[q], Tilman Grune[r,s], Nicolle Breusing[r], Andreas Simm[t], Claudio Franceschi[u], Miriam Capri[u], Duncan Talbot[v], Paola Caiafa[w], Bertrand Friguet[x], P. Eline Slagboom[y], Antti Hervonen[z], Mikko Hurme[z], Richard Aspinall[A]

[a] *Molecular Toxicology Group, Department of Biology, Box 628, University of Konstanz, 78457 Konstanz, Germany*
[b] *BioTeSys GmbH, 73728 Esslingen, Germany*
[c] *Spanish National Cancer Research Centre (CNIO), 3 Melchor Fernandez Almagro, 28029 Madrid, Spain*
[d] *DNage BV[1], Leiden, The Netherlands*
[e] *Department of Genetics, Erasmus University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands*
[f] *University of Namur, Research Unit on Cellular Biology, Rue de Bruxelles, 61, Namur B-5000, Belgium*
[g] *Research Institute for Biomedical Aging Research, University of Innsbruck, Rennweg, 10, 6020 Innsbruck, Austria*
[h] *Translational Research Center of Nutrition and Ageing, IRCCS-INRCA, Via Birarelli 8, 60121 Ancona, Italy*
[i] *Nestlé Institute of Health Sciences SA, Molecular Biomarkers, EPFL Innovation Park, 1015 Lausanne, Switzerland*
[j] *National Hellenic Research Foundation, Institute of Biology, Medicinal Chemistry and Biotechnology, Athens, Greece*
[k] *Laboratory of the Molecular Bases of Ageing, Nencki Institute of Experimental Biology, Polish Academy of Sciences, 3 Pasteur street, 02-093 Warsaw, Poland*
[l] *Ana Aslan – National Institute of Gerontology and Geriatrics, Bucharest, Romania*
[m] *National Institute for Public Health and the Environment (RIVM), Centre for Prevention and Health Services Research, P.O. Box 1, 3720 BA Bilthoven, The Netherlands*
[n] *Straticell, Science Park Crealys, Rue Jean Sonet 10, 5032 Les Isnes, Belgium*
[o] *Department of Engineering – BCE Protein Engineering, Gustav Wiedsvej 10, 8000 Aarhus, Denmark*
[p] *Life and Health Sciences, Aston Research Centre for Healthy Ageing, Aston University, Birmingham, UK*
[q] *Department for Molecular Biomedical Research, VIB, Ghent, Belgium*
[r] *Institute of Nutritional Medicine, University of Hohenheim, 70593 Stuttgart, Germany*
[s] *Department of Nutritional Toxicology, Friedrich Schiller University Jena, Dornburger Str. 24, 07743 Jena, Germany*
[t] *Department of Cardiothoracic Surgery, University Hospital Halle, Ernst-Grube Str. 40, 06120 Halle (Saale), Germany*
[u] *CIG-Interdepartmental Center "L.Galvani", Alma Mater Studiorum, University of Bologna, 40126 Bologna, Italy*
[v] *Unilever Corporate Research, Sharnbrook, UK*
[w] *Department of Cellular Biotechnologies and Hematology, Faculty of Pharmacy and Medicine, "Sapienza" University Rome, V.le Regina Elena 324, 00161 Rome, Italy*
[x] *Sorbonne Universités, UPMC Univ Paris 06, UMR UPMC CNRS 8256, Biological adaptation and ageing – IBPS, INSERM U1164, F-75005 Paris, France*
[y] *Department of Molecular Epidemiology, Leiden University Medical Centre, Leiden, The Netherlands*
[z] *Medical School, University of Tampere, 33014 Tampere, Finland*
[A] *Regenerative Medicine Group, Cranfield Health, Cranfield, UK*

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ABSTRACT

Many candidate biomarkers of human ageing have been proposed in the scientific literature but in all cases their variability in cross-sectional studies is considerable, and therefore no single measurement has proven to serve a useful marker to determine, on its own, biological age. A plausible reason for this is the intrinsic multi-causal and multi-system nature of the ageing process. The recently completed MARK-AGE study was a large-scale integrated project supported by the European Commission. The major aim of this project was to conduct a population study comprising about 3200 subjects in order 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.

* Corresponding author. Tel.: +49 7531 884035; fax: +49 7531 884033.
E-mail address: alexander.buerkle@uni-konstanz.de (A. Bürkle).

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1. Introduction

Ageing has been defined as the time-dependent decline of functional capacity and stress resistance, associated with increased risk of morbidity and mortality. Ageing is a process that affects most if not all tissues and organs of the body. Moreover, cross-talk can occur between multiple physiological systems, *e.g.* metabolic systems may influence the ageing of the immune system. The mechanisms underlying the ageing process are beginning to be unravelled at the molecular level (López-Otín et al., 2013), yet there is clear evidence 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 classical quantitative assessment of "the rate of ageing" relies on the analysis of mortality curves (Gompertz function) of populations. In other words, individuals have to be followed up until the end of their lives in order to determine their "biological age" at any time point during life. Therefore, at the level of a living individual, a reliable of assessment of the state of ageing, *i.e.* the state of the above-mentioned functional decline, and a prediction of the risk of the onset of morbidity and the residual individual life expectancy are not possible with this method.

One strategy to solve this problem is the identification of (an) age-related change(s) in body function or composition that could serve as a measure of "biological" age and predict the future onset of age-related diseases and/or residual lifetime more accurately than chronological age. Such parameters are termed "biomarkers of ageing" (Baker and Sprott, 1988). This term has been coined in analogy to biomarkers of specific chronic diseases, such as HIV infection, or biomarkers of exposure to toxins.

The American Federation for Aging Research has proposed the following criteria for a biomarker of ageing:

1. It must predict the rate of ageing. In other words, it would tell exactly where a person is in their total life span. It must be a better predictor of life span than chronological age.
2. It must monitor a basic process that underlies the ageing process, not the effects of disease.
3. It must be able to be tested repeatedly without harming the person, for example, a blood test or an imaging technique.
4. It must be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans.

The fourth of the above criteria may, however, be questioned as there are certainly some parameters whose importance for the ageing process may differ between mammalian species. One example would be telomere shortening in humans and in laboratory mice: While in human somatic tissues telomere shortening can readily be detected, this is not the case in wild-type laboratory mouse strains owing to their much greater overall length of telomeres. Therefore eliminating some candidate parameters just based on their lack of relevance in some model systems may lead to an exclusion of parameters that are potentially interesting for the human system.

It should be noted that many candidate biomarkers of human ageing have been proposed in the scientific literature but in all cases their variability in cross-sectional studies is considerable, and therefore no single measurement has proven to serve a useful marker to determine, on its own, biological age. A plausible reason for this is the intrinsic multi-causal (Holliday, 2006) and multi-system nature of the ageing process. MARK-AGE was a large-scale integrated project supported by the European Commission. The major aim of this project was to conduct a population study comprising about 3200 subjects in order 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.

2. MARK-AGE consortium

In order to tackle the scientific problem of establishing powerful biomarkers of human ageing, the MARK-AGE consortium, which consisted of 26 Partners comprising 21 non-profit organisations (universities and public research institutes), 3 small and medium sized enterprises (SMEs), and 2 large companies, was formed. The scientific groups involved are at the forefront in the field of ageing research, and some Partners are international leaders even in wider fields such as Genetics. The MARK-AGE consortium was characterised by a high degree of interdisciplinarity: The array of expertise ranged from Geriatrics, Epidemiology and Human Genetics to Clinical Chemistry, Biochemistry, Cell Biology, Immunology, Molecular Genetics, Bioinformatics and Mathematical Modelling. Such a level of interdisciplinarity is essential for the success of a project of this large scale. The lead researchers are the authors on this document.

3. The MARK-AGE strategy

In the Large-Scale Integrating Project MARK-AGE, the Partners proposed to perform a comprehensive and coherent Europe-wide population study aiming at the identification of powerful biomarkers of human ageing across a range of physiological

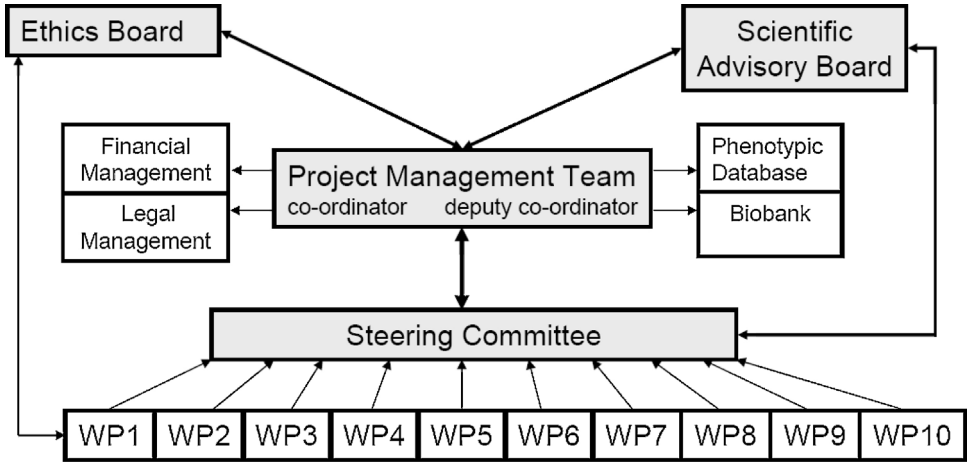

Fig. 1. Schematic representation of the management structure of the MARK-AGE project.

Table 1
MARK-AGE work packages.

Work package number	Work package title
1	Recruitment of probands and physiological markers
2	DNA-based markers
3	Markers based on proteins and their modifications
4	Immunological markers
5	Clinical chemistry, hormones and markers of metabolism
6	Oxidative stress markers
7	Emergent biomarkers of ageing from model systems and novel methodological approaches
8	Data analysis and bioinformatics
9	Dissemination and training
10	Project management and ethical issues

systems. The study population comprised of about 3200 subjects and represented several different geographical regions of Europe. The study population covered the age range of 35–74 years, as this is the time span during which prophylaxis / intervention to counter age-related diseases may be possible and promising. A wide range of candidate biomarkers was tested, including (1) "classical" ones for which data from several smaller studies have been published; (2) "new" ones, based on preliminary data obtained in small-scale studies, as well as (3) "novel" ones, based on recent research on mechanistic aspects of ageing, conducted by project Partners.

It is reasonable to assume that a combination of several biomarkers will provide a much better tool to measure biological age than any single biomarker in isolation. It has to be taken into account, though, that not all biomarkers are of equal weight. Therefore averaging all possible candidate biomarkers may not appropriate. A major task of this project was, therefore, to optimise the weighting of the different markers, by using multi-variate analysis, with the aim of reducing variance and to derive a mathematical formula that will yield a "biological age score". It should be mentioned that work performed in the context of the "MacArthur studies of successful aging" on a cohort of 171 adults aged 70–79 had already provided proof-of-concept by showing that an "allostatic load score", incorporating 10 biological markers, can be predictive of mortality risk (Seeman et al., 1995).

The MARK-AGE project provided a systematic approach: The Consortium established Standard Operating Procedures for the recruitment of subjects and processing of samples (see Moreno-Villanueva and Capri et al., this issue), as well as quality control measures (Jansen et al., this issue). It was deemed essential to recruit a new population of subjects, since previous recruitment efforts performed in many European countries neither have covered the age range of interest nor have they provided the biological materials to be studied, including cryopreserved blood cells.

The activities within the MARK-AGE project were distributed in Work Packages (Table 1)

3.1. WP1: recruitment of subjects and assessment of physiological markers

Two large groups of subjects were recruited. After exclusion of 138 hepatitis positive subjects, the first group comprised of 2262 randomly recruited age-stratified individuals from the general population (RASIG) from several different geographical regions of Europe. Equal numbers of men and women were enrolled, comprising similar 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 was thought to be display 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) (528 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 were therefore compared with their spouses, henceforth designated spouses of GEHA offspring (SGO) (305 subjects). Systematic comparison of GO and SGO cohorts should 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 display a lower biological age than SGO.

The project also took 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) or Werner's syndrome (WS). Due to the (extreme) rarity of these syndromes, only a small number of DS subjects were recruited, while biological material (serum, plasma, urine and blood) from WS patients was stored in the MARK-AGE biobank. The ageing process of DS and WS subjects is being compared with RASIG and GO/SGO subjects. It is expected that their biomarkers indicate a higher biological age, and so this comparison is expected to provide an additional validation for the biomarkers identified in the cross-sectional analysis of RASIG.

In order to ascertain the biological and analytical robustness of the measurements of candidate biomarkers, 97 donors from the whole study population have been re-sampled within 3–6 months. In such a short time period, no significant change in the biological age status of the subjects is expected; therefore an ideal biomarker essentially should yield the same results.

Finally, a limited random sample of subjects was followed-up in a small longitudinal study, compatible with the time and budgetary constraints of the project. We re-tested 12% of the recruited subjects RASIG, GO, SGO (389 subjects in total) after 3 years. It was expected that those subjects whose biomarker profile indicated an advanced biological age at baseline should display a similar or accelerated pattern at the 3-year follow-up, and vice versa for the biologically younger individuals.

From all subjects enrolled, anthropometric, clinical and demographic 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 – forced expiratory volume in 1 s (FEV1).
- Lung function – forced vital capacity (FVC).
- Five-times chair standing.
- Handgrip strength.

All subjects were asked to donate blood (55 ml) by venipuncture after overnight fasting. The blood sample was processed to obtain plasma, serum and peripheral blood mononuclear cells (PBMC). PBMC were cryopreserved and all the other components were frozen down. Buccal mucosal cells were also collected (using a kit) as well as spot urine samples (see Moreno-Villanueva and Capri et al., this issue).

3.2. WP 2: DNA-based markers

The integrity of the nuclear genome and the epigenome is of vital importance for the proper function of cells, tissues and organs. There is, however, a constant attack by exogenous and endogenous compounds and agents (including reactive oxygen species [ROS]) that can damage DNA and/or disturb epigenetic regulation. Possible consequences are mutation and dysregulation of gene expression, which either can lead to cell death or cellular senescence or to malignant transformation of the cells ultimately resulting in cancer. Protection and maintenance systems have evolved that help maintain a sustainable steady-state level of molecular damage, and these include DNA repair systems and DNA methyl transferases. Furthermore telomeric DNA undergoes attrition with each replication cycle and also as a consequence of DNA damage. A critical loss of telomere repeat sequences has been shown to prevent further cell proliferation and in some cell types induces cellular senescence.

Mitochondrial DNA is an especially vulnerable target for mutagenesis, in view of the high local levels of endogenous ROS in mitochondria. Damage and mutation of mitochondrial DNA is viewed as a major mechanism driving the ageing process (Niemi et al., 2003; Wong et al., 2009; Altilia et al., 2012). Nevertheless D-loop region contains level of heteroplasmy associated with longevity, as previously identified (Rose et al., 2007), suggesting also mtDNA variants- based mechanisms of protection (Raule et al., 2014). Further, APOE genotype, which is considered a gold standard for the genetics of longevity and was recently re-confirmed (Deelen et al., 2014), was taken into account with the basic idea to identify possible subgroups of individuals with best or worst health conditions (extreme phenotypes).

Our overarching hypothesis was that the presence of proficient systems to prevent/repair damage and mutation (Beneke and Burkle, 2007) to the nuclear genome (Reale et al., 2005; Caiafa and Zampieri, 2005; Zardo et al., 2002), including telomere shortening (Canela et al., 2007; Benetti et al., 2007; Flores et al., 2005; Gonzalo et al., 2006), and to the mitochondrial genome (Bellizzi et al., 2006; De Benedictis et al., 1999) should help retard the ageing process in many if not all tissues. Therefore these cellular functions have a potential to serve as 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 was to be correlated with the possible age-dependent expression level of some genes whose expression has been associated with ageing or longevity.
- The ageing-dependent 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 was to be addressed by quantifying the level of heteroplasmy. Special attention was paid to heteroplasmy of the mtDNA control region.
- Donors were stratified for their *APOE* genotype to correlate this with the type of ageing, *i.e.* successful or unsuccessful ageing.

3.3. WP 3: markers based on proteins and their modifications

One important physiological posttranslational modification of secreted proteins is addition of N-linked oligosaccharides (*N*-glycans). Since most *N*-glycans are on the outer surface of cellular and secreted macromolecules, they can modulate or mediate a wide variety of events in cell-cell and cell-matrix interactions crucial for the development and function of complex multicellular organisms. Because the biosynthesis of glycans is not controlled by interaction with a template but depends on the complicated concerted action of glycosyltransferases, the structures of glycans are much more variable than those of proteins and nucleic acids, and they can be easily altered by the physiological conditions of the cells. Accordingly, studying age-related alterations of the glycans could be relevant to understanding the complex physiological changes in ageing individuals (Vanhooren et al., 2010). The objective of this sub-task was to determine the changes in the blood *N*-glycome during human ageing of healthy humans and to develop methodology for profiling urine *N*-glycans (Vanhooren et al., 2007).

The apolipoprotein J/Clusterin (ApoJ/CLU) is a highly conserved multifunctional glycoprotein. Amongst its multiple physiological functions, this protein is a chaperone that stabilizes stressed proteins in a folding-competent state. Previous work had shown that ApoJ/CLU is associated with human ageing and with ageing of human cells *in vitro*, and that its level is increased in patients with type II diabetes, coronary heart disease, and myocardial infarction. Therefore ApoJ/CLU may represent a valuable ageing biomarker.

Non-enzymatic protein glycation is a common posttranslational modification of proteins *in vivo*, resulting from reactions between glucose and amino groups on proteins; this process is termed "Maillard reaction" and leads to the formation of Advanced Glycation Endproducts (AGEs). During normal ageing, there is accumulation of AGEs of long-lived proteins such as collagens and cartilage. AGEs, either directly or through interactions with their receptors, are involved in the pathophysiology of numerous age-related diseases (Simm et al., 2014), such as cardiovascular and renal diseases and neurodegeneration. However, in a cohort study on human ageing, the correlation of AGEs with human age remained debatable. By analysing overall parameters of AGEs as well as specific AGEs, it was to be determined if these modifications correlate with age independently of disease and if there are gender differences (Scheubel et al., 2006; Simm et al., 2004).

It is well known that levels of oxidised proteins increase with age, due to increased protein damage induced by ROS, decreased elimination of oxidised protein (*i.e.* repair and degradation), or a combination of the two. Since the proteasome is in charge of both general protein turnover and the selective removal of oxidized protein, its fate during ageing has received considerable attention, and evidence has been provided for an impairment of proteasome function with age in different cellular systems (Chondrogianni and Gonos, 2010; Baraibar and Friguet, 2013), including human PBMC (Chondrogianni et al., 2003, 2005; Carrard et al., 2003; Friguet, 2006). Apart from to being degraded, certain oxidised proteins can be repaired. However, repair is limited to the reversion of a few oxidative modifications of sulphur-containing amino acids, such as the reduction of methionine sulfoxide by the methionine sulfoxide reductase (Msr) system. Msr activity is known to be impaired during ageing and replicative senescence. Therefore, the status of both proteasome and Msrs A and B in human PBMC from the recruited

donors of different ages was to be assessed. These parameters had previously been shown to be key players in oxidised protein degradation and repair and to exhibit a declining activity with age (Picot et al., 2004, 2007). These protein maintenance systems, viewed as potential biomarkers of ageing, were to be monitored at the levels of enzymatic activity, protein expression, and RNA expression.

The following research tasks have specifically been addressed:

- Analysisof the *N*-glycomic changes in glycoproteins from blood of all donors. Urine glycoprotein changes were to be 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.

3.4. WP 4: immunological markers

Thymic output is known to decline with age; furthermore the rate of decline is dependent on gender, with greater thymic output for longer in females compared with males (Aspinall et al., 2007). Females often develop better immune responses than males, which may relate to their longer lifespan. Within MARK-AGE, signal joint T-cell receptor rearrangement excision circles (sjTREC) were assessed as a candidate biomarker of ageing and thymic involution, by analysing sjTREC levels in individuals at various ages (Aspinall et al., 2007).

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 CMV infection, have been shown to be associated with shorter life expectancy. Auto-immune responses may also restrict the diversity of immune responsiveness to foreign antigens. We therefore evaluated long-term and short-term immunological memory and autoimmune responses as potential biomarkers of ageing (Almanzar et al., 2005; Kovaiou et al., 2007; Weinberger et al., 2007; Herndler-Brandstetter et al., 2005).

In vitro, two types of senescence have been described. One is telomere-dependent replicative senescence and the second is stress-induced premature senescence (SIPS) (Sikora et al., 2014). In view of previous results we hypothesised that during ageing, chronic antigenic load as well as oxidative stress may 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 studied DICD and AICD in T cells from donor samples (see Sikora et al., this issue).

The following research tasks have specifically been 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 was 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.

3.5. WP 5: 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 Partners (Al-Delaimy et al., 2006; Rezzi et al., 2007a,b; Kochhar et al., 2006; Heijmans et al., 2006; Mooijaart et al., 2006; Hurme et al., 2005, 2007; Rontu et al., 2006; Lehtimaki et al., 2007).

The following candidate biomarkers have specifically been addressed:

Systemic metabolism and toxicity parameters

- Blood urea nitrogen and creatinine, used for the evaluation of renal function.
- Metal binding proteins including transferrin, ferritin, α_2-macroglobulin and ceruloplasmin, in order to complement metal ion determinations (see below).
- Fasting glucose and fasting insulin as a measure for glucose homeostasis, insulin resistance and diabetic conditions.
- Glycosylated hemoglobin (A_{1C}) as a measure for the long-term systemic glucose load, in order to detect (pre) diabetic conditions.
- Some basic/reference parameters, including albumin and serum protein concentration.

Fatty acid and cholesterol metabolism parameters

- Fasting triglycerides and free fatty acids were measured to detect metabolic disorders in lipid metabolism.
- Total cholesterol, HDL and LDL-cholesterol were measured (together with triglycerides) for risk assessment of cardiovascular diseases.
- Concentrations of lipoprotein particle size classes by NMR.

Systemic inflammation parameters

- C-reactive protein (CRP), homocystein, uric acid and fibrinogen are inflammatory markers associated cardiovascular disease and hypertension.
- Serum amyloid A and P, and pentraxin 3 were amongst the acute phase proteins studied.
- Adiponectin is correlated with an anti-inflammatory state and suppresses metabolic derangements that may result in type II diabetes, obesity, atherosclerosis and non-alcoholic fatty liver disease.

Additional candidate biomarkers

- Testosterone, the principal male sex hormone whose levels are known to decline gradually with age in males.
- Prostate specific antigen (PSA) was measured for the detection of (pre) neoplastic processes in the prostate and prostate cancer in particular.
- Vitamin D, a recently identified promising candidate related to ageing and several chronic diseases, was also studied.
- Dehydroepiandrosterone sulfate (DHEAS) is known to decline with age and is a classical candidate biomarker of human ageing (Lane et al., 1997).

Novel biomarkers to be derived from metabonomics analysis

- Nuclear Magnetic Resonance (NMR)-based metabolic profiling of serum/plasma samples and urine samples from probands. NMR profiles display a set of resonances arising from major low-molecular weight molecules, such as ketone bodies, organic acids, amino acids, and aromatic metabolites (Oostendorp et al., 2006; Rezzi et al., 2007a,b; Ramadan et al., 2007)

3.6. WP 6: oxidative stress markers

It has been postulated that oxidative stress is causal for the ageing process. Oxidative stress refers to an imbalance between ROS formation and antioxidant defence. In human beings large amounts of oxidants are formed in various physiological metabolic reactions and even a wider variety of pathophysiological conditions. The body is able to respond to such enhanced oxidant formation with compensatory antioxidant reactions. These reactions are also complex and a large variety of different enzymes are involved. If antioxidant protection is insufficient, oxidative stress with an enhanced formation of oxidative stress markers occurs.

Oxidative damage accumulation in macromolecules has been considered causative for cellular damage and pathology. Such damage seems to be closely related to the ageing process. Although the relationship between oxidative damage and the ageing process has been established in various model systems, only few studies reported a systematic analysis of oxidative stress parameters in healthy humans related to age of individuals (Pandey and Rizvi, 2010; Gil et al., 2006).

The purpose of this work package was to analyse a set of parameters of oxidative stress parameters (Gil et al., 2006), vitamins and trace elements (Mazzatti et al., 2007; Malavolta et al., 2006; Mocchegiani et al., 2006) in human blood, serum, urine and buccal mucosa cells. Preference was 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 have specifically been addressed:

- Malondialdehyde.
- Carbonylated and nitrated proteins.
- Oxidation of LDL.
- NO metabolic-pathway products $(NO_x)_x$.
- Isoprostanes.
- Cellular glutathione.
- Vitamin content (α-tocopherol, α-carotene and ascorbate) of serum and buccal mucosal cells.
- Trace elements (Zn, Cu, Se and Fe) in blood/serum.

3.7. WP 7: 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 prolonged periods of time until their chronological age deviates from their biological age. To overcome this problem, we adopted 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 used both established and novel approaches to search for biomarkers of ageing in an iterative process, where markers derived from models would inform *in vivo* biomarker searches.

(a) Model Systems

Progeroid mouse models

Progeroid syndromes are rare disorders with premature ageing and a shortened life expectancy. These conditions are characterized by extremely accelerated ageing, showing many hallmarks of normal ageing including cessation of growth, liver, kidney and bone abnormalities, retinopathy, hearing loss, sarcopenia, neurodegeneration, sensitivity to UV light, and a premature aged appearance due to kyphosis, baldness, loss of subcutaneous fat, and dry wrinkled skin. Cockayne syndrome (CS), Hutchinson-Gilford syndrome, Werner's syndrome (WS), Bloom's syndrome, and trichothiodystrophy are all autosomal recessive disorders with progeroid symptoms. Although some differences exist in the pathology of these conditions it is striking that the causal factor of all these syndromes lies in impaired genome maintenance due to DNA repair deficiencies or genome instability. One aim of the project was the identification of biomarkers of ageing in CS, a rare human disorder, in which patients suffer from segmental but *bona fide* accelerated ageing. The mean age at death of CS patients is 12.5 years. There is currently no treatment for CS and related disorders available and the clinical management of patients is purely supportive. CS is an autosomal recessive disorder caused by mutations in the *CSA* or *CSB* genes, which are involved in transcription-coupled nucleotide and base excision repair (TCER). The TCER sub-pathway selectively removes lesions from the transcribed strand that actually block transcription. As such this process is important for promoting recovery of the vital process of transcription and thus cellular survival from transcription-blocking DNA lesions. One of the objectives of the project was to apply the biomarkers studied in Work Packages 2 through 6 to progeroid patients, thereby determining to which extent premature and normal ageing resemble each other and their suitability to identify ageing features independent of chronological age.

To understand the aetiology of CS and other DNA repair disorders such as trichothiodystrophy (TTD) and the cancer-prone condition *xeroderma pigmentosum* (XP), an extensive collection of DNA repair-deficient transgenic mice had previously been generated, many of which mimic the hallmarks of the corresponding human repair syndrome. These mice display either a strong cancer predisposition (XP-like) or many features of premature ageing, or a combination. Although the $Csb^{m/m}$ mouse model reliably reflects the repair defect and UV-sensitive phenotype of the patient, animals show relatively mild growth retardation and neurological abnormalities, accompanied by age-related retinal degeneration. Interestingly, when TCER-defective $Csb^{m/m}$ mice were crossed with completely NER-deficient $Xpa^{-/-}$ animals double mutants phenocopy human CS surprisingly well, including its age-related

pathology. Although $Csb^{m/m}/Xpa^{-/-}$ pups are devoid of any overt embryonic developmental phenotype, they display severe postnatal growth retardation, impaired psychomotor development, ataxia, progressive cachexia, and kyphosis. Loss of retinal photoreceptors is also further accelerated, as compared to $Csb^{m/m}$ animals. Moreover, most $Csb^{m/m}/Xpa^{-/-}$ newborns die during or shortly after birth, whereas animals that survive birth stress do not survive beyond three weeks. Exploiting the genetic and environmentally fully defined mouse system these and other mouse mutants for XP, XP/CS and TTD provided a convenient tool to deduce specific biomarkers in various organs/tissues including serum. Within MARK-AGE a set of mouse biomarkers for ageing was developed and evaluated, using transcriptomics, immunohistochemistry and serum/urine markers in prematurely ageing mouse models with different life span and ageing-related pathology to deliver universal markers of ageing. Biological material from genetically and environmentally controlled, (histo) pathologically well-defined cohort studies with NER-deficient mouse models, served to identify parameters that were expected report on the biological age of the animals and/or the onset and progression of ageing-related pathology in various tissues (*e.g.* liver, brain) (van der Pluijm et al., 2007; de Boer et al., 2002; Rossi et al., 2007; Niedernhofer et al., 2006).

Stress induced premature senescence

There are several pathways activating cellular senescence; these include telomere uncapping, DNA damage, oxidative stress and oncogene, amongst others (Ben-Porath and Weinberg, 2005). Normal human diploid fibroblasts cultured *in vitro* irreversibly stop dividing after a certain number of cumulative population doublings in a process known as replicative senescence (Hayflick, 1965). This limited proliferative life span has been observed in many other eukaryotic cell types and has been interpreted as a manifestation of cellular ageing. Random metabolic modifications appear within these cells over time, leading to random damage of the cellular components. These damaged cellular components are not completely eliminated or repaired and therefore accumulate with time and progressively impair cellular functions. Cellular senescence can be also regarded as a permanently maintained DNA damage response state (von Zglinicki et al., 2005). ROS are important contributors to the ageing process and we have confirmed the similarities between replicative senescence and stress induced premature senescence (SIPS) (Dumont et al., 2000; Dierick et al., 2002; Pascal et al., 2005). In MARK-AGE, we used SIPS to "age" T and B cells from the subjects recruited in WP1 and searched for novel biomarkers using genomic array and proteomic approaches described below (Debacq-Chainiaux et al., 2005; Frippiat et al., 2001).

- Novel methodological approaches

Analysis of miRNAs

MicroRNAs (miRNAs) are small, abundant non-coding RNA molecules of about 21–23 nucleotides that have been shown to affect a broad spectrum of biological activities. Interestingly, there is evidence that a remarkably large proportion of genes (>30%) are subject to microRNA-mediated regulation. In general, miRNAs function post-transcriptionally by inhibiting translation from specific target mRNAs. Up to now, about 600 miRNAs have been characterized in humans. These small RNA molecules were thought to contribute to ageing of *C. elegans*. It has been previously shown that miRNA cause a general reduction of message-specific translational inhibition during ageing. Reducing the activity of a specific miRNA lin-4 shortened life span and accelerated tissue aging, whereas overexpressing lin-4 or reducing the activity of lin-14 extended life span of *C. elegans*, frequently used as a model system for mammalian ageing (Boehm and Slack, 2005). Studies on miRNA expression levels in tissues of young and old mice showed the differential and clearly tissue specific expression of some miRNAs (Smith-Vikos and Slack, 2012). On the level of cells, it was also shown that such differential expression can directly influence cellular ageing. miR-21 was found up-regulated by replicative and stress-induced senescence in human endothelial cells. miR-21 over-expression reduces the replicative lifespan, while stable knock-down extends the replicative lifespan of these endothelial cells (Dellago et al., 2013). On the organ level, it is clear that not all miRNAs that are up- or down-regulated during ageing necessarily play crucial roles during ageing. As no "key regulator" on tissue ageing was identified yet in mammalians, one has to clarify which miRNAs are activated or repressed especially in degenerative disease contexts and which are really associated with aging per se. Therefore we are evaluating miRNA expression as a "novel" biomarker of ageing using the leukocytes of subjects recruited in WP1.

Phage antibodies against novel markers of endothelial and T cell ageing

The phage display antibody library technology has been found to be a useful method to isolate antigen-specific antibody fragments, since the repertoire of antibody specificities is broad and since it bypasses the need of immunization. When applied as a discovery tool, the phage display technology can be considered a complementation to traditional proteomic approaches using 2-D gel electrophoresis and mass spectrometry, which quite often have problems in identifying proteins which are very hydrophobic (Gonzalez-Dosal et al., 2006; Jensen et al., 2003). During ageing, both in vivo and in vitro, changes in the proteomic profile are observed. By performing subtractive selection of recombinant antibodies binding to e.g. endothelial cells allowed to age *in vitro*, where young cultured endothelial cells is applied as competitor, antibodies binding potential biomarkers of ageing can be obtained (Boisen et al., 2010; Boisen and Kristensen, 2010)

Such approaches have enabled the development of a panel of antibodies recognizing *in vitro* ageing human endothelial cells during the previous EU project Proteomage, in particular the secretome of endothelial cells. With age there is a decrease in the ability to form new blood vessels, which is a biomarker of ageing. The purpose of the work in the MARK-AGE project was to evaluate the *in vivo* significance of endothelial secretome biomarkers identified from *in vitro* models for their *in vivo* relevance by screening plasma from subjects recruited in WP1 for these markers. More specifically it was found that the intermediate filament protein, vimentin, is found in the serum. As studies within the EU funded project, Proteomage, established that extracellular vimentin can exert functional changes to the ability to form new blood vessels, it has been of particular interest to see if there is an age specific correlation with the amount of vimentin in serum. This, in part, might explain why in general older people exhibit decreased ability to form new blood vessels. Using a battery of antibodies raised against endothelial progenitor cells, it was further proposed to evaluate whether the number of endothelial progenitor cells in serum qualifies as a biomarker of ageing (Bertelsen et al., 2014; Williamson et al., 2012). This again could have implications for the generally decreased ability of older subjects to form new blood vessels.

Microarray and proteomics

Genomics and proteomics offer the opportunity for an unbiased systematic discovery route for novel biomarkers and are becoming increasingly popular (Griffiths et al., 2002; Griffiths et al., 2006; Aldred et al., 2006; Grant et al., 2007). Nevertheless, only few groups have undertaken proteomic studies of either plasma proteins of mononuclear cells in healthy human ageing. The first of these, published by Thambietty et al. in 2010, described a differential plasma protein pattern between 57 older adults with and without amyloid deposition in the brain (Thambisetty et al., 2010). Subse-

quently, differential expression of ApoE and antioxidant proteins was observed in the plasma of 10 Japanese supercentenarians compared with 10 young people (Miura et al., 2011). In 2012, some of us described alterations in transferrin glycosylation during healthy ageing (Dunston et al., 2012). The advantages of applying such an approach in the MARKAGE population is the greater power to evaluate the validity of novel biomarkers discovered through proteomics when compared with small sample size discovery programmes. After the completion of MARKAGE, one other study has analysed by ELISA the levels of protein biomarkers that were not discovered using proteomics in plasma during healthy ageing in comparison with older adults that develop frailty syndromes. These authors showed that higher levels of transferrin fibrinogen and interleukin-6 were associated with frailty status and frailty score (Darvin et al., 2014).

Ever since the concept of MARK-AGE has been developed microarray has been used extensively to characterise ageing-related changes in gene expression. Indeed, the systematic analysis of miR, single nucleotide polymorphisms and deep sequencing approaches have led to further insight into expression changes in specific cell types (Laurie et al., 2012; Smigielska-Czepiel et al., 2014; van der Brug et al., 2010). The latter are by necessity cross-sectional studies. The approach taken by MARK-AGE was to investigate expression changes within unique accelerated models of ageing the consortium had access to. Systematic microarray analysis of progeroid mutants has already yielded new potential biomarkers: using full-genome microarray analysis some of us have recently identified a 'survival' response in the progeroid mouse models directed by down-regulation of the IGF1 somatotrophic axis that boosts antioxidant defence, down-regulates metabolism and redirects energy resources from growth and development to protection, maintenance and repair. This adaptive switch aims to slow down ageing-related pathology and postpones death, thereby promoting successful ageing. It is constitutively turned on in the repair-compromised mouse mutants as a futile attempt to extend lifespan and explains their dwarf phenotype. In normal mice the same principally beneficial response can be transiently triggered by chronic exposure to DNA-damaging agents and ROS-producing compounds. We hypothesise that normal ageing is also associated with a similar response due to age-dependent accumulation of damage. The existence of such a response allows predictions for shifts in levels of specific proteins, activities, pathways and metabolites that could serve as biomarkers. As the proteome is far more extensive than the transcriptome, it offers a richer source of potential biomarkers but also poses increased problems in terms of dynamic range, particularly in plasma. This is being addressed using quality-assured subfractionation steps and restricted IPG range in the first dimension. In MARK-AGE, proteomics was to be adopted in accordance with HUPO guidelines, in the search for biomarkers in plasma from subjects recruited in WP1, in CS/progeria and in T and B cells subjected to SIPS in subjects recruited in WP1. The identification of putative markers was to be confirmed by sequence analysis and their validation as biomarkers of ageing confirmed by alternative approaches such as ELISA where possible. In view of the strong parallels between the mouse mutants and the human syndromes, down-regulation of the IGF1 somatotrophic axis biomarkers is likely to be instrumental for identification of corresponding markers in human patients and even normal ageing and will be evaluated in WP1 subjects.

3.8. WP 8: data analysis and bioinformatics

In view of the large amounts of clinical and biochemical data, which have been collected in the framework of the MARK-AGE project an appropriate and coherent strategy of data analysis and model building is mandatory.

In order to extract a robust set of biomarkers of human ageing and to derive a model for healthy ageing, the following tasks were performed:

- Dataproperty analysis. Partial knowledge about suspected correlations between measurements is available and being used to judge the noise ratio within some of these measurements using classical statistical techniques. We also applied correlation measures to identify additional relationships between measurements. Repeated sampling, *i.e.* obtaining samples after 6 months from 97 subjects, was done to further investigate biological and analytical variability in the measurements.
- Modelling. Both, statistical models as well as machine learning/data mining methods were to be used in order to build models aiming to predict biological age from the available measurements. We used classical techniques, such as regression analysis but also aim to introduce additional knowledge (monotonicity) to improve those models. Neural Networks and Decision/Regression Trees were to be used to find more local relationships within the data, for instance revealing properties that are relevant only for a subset of the chosen population.
- Variance reduction. Through dimensionality reduction techniques (principal component analysis and others) we aim at reducing the number of required measurements while, at the same time, reducing the variance in the predictions generated. Machine Learning offers ensembles of models for this purpose, which allows combining different, diverse predictors to generate models with lower variance.
- Clustering/visualisation. We expect to discover previously unknown or unexpected relationships in the data that define successful ageing. Data visualisation techniques and interactive methods such as visual clustering models may help uncover some of these relationships. It is expected that some measurements will have higher correlations with the biological age than others in parts of the population. Finding such clusters is further helping reduce variance in the generated models since we will be able to better model characteristics of subgroups.

3.9. WP 9 and WP 10

WP 9 and WP 10 were dedicated to dissemination, training, project management (Fig. 1) and ethical issues.

4. Discussion

Biomarkers of human ageing are urgently needed for variety of reasons, including the identification of individuals at high risk of developing age-associated disease or disability. This would prompt targeted follow-up examinations and, if available, prophylactic intervention (*e.g.* changes in lifestyle) or early-stage treatment of age-related disease. Furthermore, the availability of powerful biomarkers would allow the assessment of the efficacy of forthcoming pharmacological and other interventions (including optimisation of micronutrient intake and other dietary components or physical activity) currently being developed with the aim to lower the risk of age-associated disease even in individuals without accelerated ageing.

In view of the rapidly increasing average life expectancy of human beings world-wide, the prevalence of age-related diseases is likely to increase as well. This necessitates effective new strategies for prevention and early diagnosis of such conditions.

It should be noted that different types of biomarkers have been envisaged: (1) "neutral" markers of age (also called markers of "chronological ageing") possibly lacking the power of directly predicting disease risk, as the underlying physiological change may *per*

se be harmless and without functional compromise; (2) markers of (global) risk of age-related diseases. Those in category #2 are of obvious and direct interest. Nevertheless, the usefulness of those in category #1, especially as a combination of markers, should not be dismissed, as they could reflect the rate of ageing that has prevailed over a certain time period in the past and the cumulative change it has produced in the body. The detection of a previous faster-than-normal rate of ageing in a given individual should also be alarming and call for additional diagnostic and preventive measures.

Disclaimer

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