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MARK-AGE standard operating procedures (SOPs): A successful effort

María Moreno-Villanueva[a,*,1], Miriam Capri[b,1], Nicolle Breusing[c], Anne Siepelmeyer[d], Federica Sevini[b], Alessandro Ghezzo[h], Anton J.M.de Craen[e], Antti Hervonen[f], Mikko Hurme[f], Christiane Schön[d], Tilman Grune[c,g], Claudio Franceschi[b], Alexander Bürkle[a]

[a] Molecular Toxicology Group, Department of Biology, University of Konstanz, 78457 Konstanz, Germany
[b] DIMES-Department of Experimental, Diagnostic and Specialty Medicine, CIG-Interdepartmentall Centre "L.Galvani", Alma Mater Studiorum, University of Bologna, 40126 Bologna, Italy
[c] Institute of Nutritional Medicine, University of Hohenheim, 70599 Stuttgart, Germany
[d] BioTeSys GmbH, 73728 Esslingen, Germany
[e] Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, The Netherlands
[f] School of Medicine, University of Tampere, 33014 Tampere, Finland
[g] German Institute of Human Nutrition, 14558 Nuthetal, Germany
[h] National Association "Famiglie di Persone con Disabilita Affettiva e/o Relazionale" (ANFFAS) Onlus, Macerata, Italy

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ABSTRACT

Within the MARK-AGE project, a population study (3337 subjects) was conducted to identify a set of biomarkers of ageing which, as a combination of parameters with appropriate weighting, would measure biological age better than any single marker. The MARK-AGE project involves 14 European countries and a total of 26 research centres. In such a study, standard operating procedures (SOPs) are an essential task, which are binding for all MARK-AGE Beneficiaries. The SOPs cover all aspects of subject's recruitment, collection, shipment and distribution of biological samples (blood and its components, buccal mucosa cells or BMC and urine) as well as the anthropometric measurements and questionnaires.

1. Introduction

Across all European countries, the number of elderly people is increasing steadily. Therefore, it is becoming more and more important to define a reliable method of assessment of the state of ageing, which has not been possible with the available techniques. The MARK-AGE strategy to solve this problem is the identification of an age-related change in body function or composition that could serve as a measure of "biological age" and which predict the risk of onset of age-related diseases more accurately than chronological age does. Such parameters are termed "biomarkers of ageing".

The MARK-AGE project involves researchers from 14 European countries: Austria, Belgium, Denmark, Finland, France, Germany, Greece, Italy, the Netherlands, Poland, Romania, Spain, Switzerland and the United Kingdom. A total of 26 "Beneficiaries" (i.e. the participating institutions or companies) collaborated with the aim of identifying powerful "biomarkers of ageing" (Table 1). The range of candidate biomarkers to be tested includes (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 (Vanhooren et al., 2007; Garagnani et al., 2012; Dall'Olio et al., 2013; Collino et al., 2013). In consideration of the huge amounts of samples to be collected SOPs need to be defined concerning proband recruitment, sampling and processing of body fluids. A SOP is a set of written instructions that document how the involved recruiting and study staff executes the tasks and which materials are used. These detailed instructions include step-by-step details of the processes and provides instructions in order to perform the task in a consistent manner. SOPs are an essential component assuring consistency in the quality of data collection as it provides the staff with the information to perform their job properly and in a standardized manner following study requirements. The credibility of human studies that are not conducted in accordance with SOP might be compromised. Therefore the MARK-AGE Consortium dedicated the first year to elaborate

* Corresponding author. Tel.: +49 7531 884414; fax: +49 7531 884033.
E-mail address: maria.moreno-villanueva@uni-konstanz.de (M. Moreno-Villanueva).
[1] These authors contributed equally to this work.

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Table 1
List of MARK-AGE partners with a short description of their tasks.

Partnernumber	Beneficiary name	Country	Task
1	Universitaet Konstanz	Germany	Co-ordinator and analysing centre (PARP activity and DNA repair)
2	BioTeSys GmbH	Germany	Recruiting and analysing centre (VitC, VitE, Gluthatione)
3	Fundación Centro Nacional de Investigaciones Oncológicas Carlos III	Spain	Analysing centre (telomere length)
4	DNage B.V.	The Netherlands	Recruiting centre
5	Erasmus Universitair Medisch Centrum Rotterdam	The Netherlands	Prematurely ageing mouse models
6	Facultés Universitaires Notre-Dame de la Paix de Namur	Belgium	Recruiting centre
7	Imperial College of Science, Technology and Medicine	UK	Changed to Cranfield (Nr. 27)
8	Oesterreichische Akademie der Wissenschaften	Austria	Recruiting and analysing centre (measles, influenza A and B, tetanus IgG antibodies and number of cells producing INF after Influenza and CMV stimulation)
9	Istituto Nazionale Riposo e Cura per Anziani	Italy	Analysing centre (intracellular and extracellular trace elements)
10	NESTEC SA	Switzerland	Analysing centre (identification of metabolites using Magnetic Resonance Spectroscopy)
11	National Hellenic Research Foundation	Greece	Recruiting and analysing centre (serum levels of polipoprotein J)
12	Instytut Biologii Doświadczalnej im. M. Nenckiego PAN	Poland	Recruiting and analysing centre (Activation- and DNA Damage-induced cell death and
13	Institutul National de Gerontologie si Geriatrie Ana Aslan	Romania	Analysing centre (LDLox and NOx)
14	Rijksinstituut voor Volksgezondheid en Milieu	The Netherlands	Analysing centre (clinical chemistry)
15	StratiCELL Screening Technologies SA/NV	Belgium	Analysing centre (cytokines)
16	Aarhus Universitet	Denmark	Analysing centre (intermediate filament protein vimentin)
17	Aston University	UK	Analysing centre (enolase and thioredoxin 1 on CD4+ cells and transferrin residues)
18	Vlaams Instituut voor Biotechnolgie vzw	Belgium	Analysing centre (*N*-glycans)
19	Universitaet Hohenheim	Germany	Biobank and analysing centre (lycopene, carotinoide, cysteine, gamma tocopherol, VitA, VitC, uric acid, VitE, total glutathione, malondialdehyde, protein carbonyls)
20	Martin-Luther Universitaet Halle-Wittenberg	Germany	Analysing centre (advanced glycation end products (AGEs) and micro-RNA (miRNA) pattern)
21	Alma Mater Studiorum – Università di Bologna	Italy	Recruiting and analysing centre (DNA methylation status, APOE genotype and levels of heteroplasmy in mtDNA)
22	Unilever UK Central Resources Limited	UK	Analysing centre (urinary and plasma 8-isoprostane, urinary creatinine and serum adiponectin)
23	Università degli Studi di Roma "La Sapienza"	Italy	Analysing centre (DNMT, PARP 1 and 2 expression and methylation)
24	Université Pierre et Marie Curie – Paris 6	France	Analysing centre (proteasome activity)
25	Academisch Ziekenhuis Leiden – Leids Universitair Medisch Centrum	The Netherlands	Recruiting and analysing centre (cholesterol and triglycerides in HDL, HDL1, HDL2, LDL, LDL1, LDL2, VLDL, VLDL1, VLDL2)
26	Tampereen Yliopisto	Finland	Recruiting and analysing centre (cell free DNA in serum)
27	Cranfield University	UK	Analysing centre (T cell receptor excision circles (TRECs) as biomarker of thymus output)

standardized protocols and procedures. Further, the use of SOPs was reviewed and re-enforced by the coordinator regularly, The MARK-AGE project is described in detail in a separate manuscript (see Bürkle and co-workers, this issue).

2. Material and methods

2.1. Recruitment of subjects

Two large groups of subjects were recruited, i.e. (1) randomly recruited age-stratified individuals from the general population covering the age range 35–74 years ("RASIG") 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 EU GEHA project (Genetics of Healthy Ageing http://www.geha.unibo.it/). For genetic reasons such individuals ("GEHA offspring") are expected to age at a slower rate. They were recruited together with their spouses ("SGO" as controls of the shared environment). (3) A small number of patients with progeroid syndromes (Cockayne, Werner and Down syndromes) were also included in the study. Prior to recruitment of subjects each Beneficiary involved obtained the local Ethics Committee approval. Exclusion criteria were foreseen: (i) self-reported seropositivity for HIV, for HBV (except seropositivity by vaccination) and HCV; (ii) measured seropositivity for HBV and HCV (iii) presence of a diagnosed cancer disease and current use of anti-cancer drugs or glucocorticoids (chronic treatment); (iv) less than 50% of lifetime spent in country of residence; or (v) inability to give Informed Consent or (vi) any acute illness (e.g. common cold) within seven days preceding blood collection (see Buerkle et al.; Capri and Moreno-Villanueva et al., this issue)

2.1.1. Ethical approval

The MARK-AGE study was carried out in accordance with the declaration of Helsinki, which is the accepted basis for clinical study ethics, and must be fully followed and respected by all engaged in research on human beings. 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" (Supplementary materials). 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, Greek, Italian and Polish. Before

starting the interview and the examination of the subjects, each potential subject was informed in detail about the study procedures and written Informed Consent was obtained.

2.1.2. Questionnaires

The questionnaires, which were filled out by the subject and a trained interviewer, allow standardised documentation of biographic information and health status of the MARK-AGE subjects. The cognitive tests incorporated in the questionnaire provide a score that is commonly accepted in the scientific community, as is documented by many publications. These questionnaires were first written in English and then translated to the respective national languages. The questionnaires consist of two parts, one to be filled out by the subject at home before the interview, and the other to be completed by the interviewer at the time of the examination. The extensive questionnaires capture demographic information, lifestyle, cognitive status, mood, health status, and anthropometric measurements (body mass index, waist and hip circumference, blood pressure at rest, heart rate at rest, lung capacity, near vision, five-times chair standing and handgrip strength among others) (Supplementary materials). People over 65 years of age were also tested with standardized mini-mental state examination (SMMSE) (Molloy et al., 1991) and activities of daily living (ADL) (Kempen et al., 1996). Further, a specific questionnaire was developed by beneficiary # 21 for individuals of different ages affected by Down Syndrome and enrolled in this project as "accelerated ageing cohort" (see Capri and co-workers, this issue). In particular, specific cognitive tests included in the general questionnaire are briefly described below:

2.1.3. Cognitive tests

2.1.3.1. 15-Picture learning test. Immediate and delayed memory function was assessed by the 15-picture learning test (15-PLT). Subjects were shown 15 pictures of well-known items and then asked to recall as many as possible. The test was repeated three consecutive times and after 20 min. Outcome parameters were the number of correct pictures after each trial and after 20 min (delayed recall). The total number of correct answers after three trials was defined as the immediate recall. Furthermore, the number of incorrect pictures was reported for each trial. A low score indicates worse cognitive performance (Brand and Jolles, 1985).

2.1.3.2. Stroop-colour-word-test. The Stroop-colour-word-test (Stroop) was used to test selective attention. The test involves three parts that displayed 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; for example, "green" printed in blue letters, where the subject is to say "blue". Performance on part 3 is determined for a large part by the time needed to discard irrelevant but very salient information (verbal), in favour of a less obvious aspect (colour naming), also known as cognitive interference. The main outcome variables ware the times needed for each of the three test parts, a higher score therefore indicates worse performance (Stroop, 1935).

2.1.3.3. Digit-symbol substitution task. The digit-symbol substitution task (DSST) was used to assess processing speed. In the DSST, digits were presented and the subjects were asked to write the corresponding symbols in a blank space according to a given key. Outcome parameter was the number of correct digit-symbol combinations within 90 s. A low score indicates worse cognitive performance (Lezak et al., 2004).

2.1.3.4. Zung self-rating depression scale. The Zung self-rating depression scale is a short self-administered survey to quantify the depressed status of a patient. There are 20 items on the scale that rate the rating affective, psychological and somatic symptoms associated with depression. There are ten positively worded and ten negatively worded questions. Each question is scored on a scale of 1 through 4 (based on these replies: "a little of the time", "some of the time", "good part of the time", "most of the time"). Scores on the test range from 20 through 80. A higher score represent a more depressed status (Zung, 1965).

2.2. Questionnaire for down syndrome

The questionnaire comprises two parts: part I equal to that adopted on other MARKAGE populations (demographic information, lifestyle, health status, and anthropometric measurements); part II containing a standardised battery of 15 tests to evaluate different domains such as behaviour and neuropsychological features, memory and language. Aberrant Behaviour Check List (Aman et al., 1985); Adaptive Skills Vineland Scale (Sparrow et al., 1986), Visual Object Spatial Perception (Rapport et al., 1998); Weschler Intelligence Scale for Children III subtests (Wechsler, 1991) were part of test battery among others and also included in a previous work where age-related changes of adaptive and neuropsycological features in persons with DS were assessed (Ghezzo et al., 2014).

2.3. Biobank

The MARK-AGE Biobank was embedded at the Institute of Biological Chemistry and Nutrition at the University of Hohenheim, Germany, within the facilities of one MARK-AGE Beneficiary. The Biobank was aimed to provide the recruitment centres with standardized material for samples collection, manage samples income from the recruitment centres, sample outcome to the analytic partners, sample re-labeling, sample splitting, sample storage and organization of an internal data base for sample tracking. The central role of the Biobank was implemented in the Mark-Age SOPs.

The Biobank hardware consisted of three ultra-low temperature freezers (−80 °C, New Brunswick Scientific, Enfield, USA) and four nitrogen tanks (−196 °C, Air Liquide, Paris, France), which assured the permanent freezing conditions for the samples and the structured storage of several thousands of samples. In all freezers and tanks temperature fluctuations were permanently recorded. Furthermore, the filling level of the nitrogen tanks was documented. Freezers were additionally equipped with a CO_2 backup system holding conditions for about 2 days. A central current generator at the facility guaranteed the maintenance of the freezer function after a local power failure. All freezers were equipped with an alarm system, automatically informing maintenance staff and scientists (via mobile phone) about potential troubles. The incoming and outgoing samples, as well as the storage place, were registered in a central Biobank computer. The data were daily uploaded to a central server in a different building in order to avoid any fatal data hazard.

2.4. Recruiting centres

The recruiting centres were supplied with collection material by the Biobank: eight blood monovettes (Sarstedt, Germany) ranging from 2.7 to 9 ml containing lithium-heparine, EDTA or none of these as anti-coagulants, 29 cryo tubes ranging from 0.5 to 15 ml (Greiner Bio-one, Germany) and one BMC kit (BioTeSys Germany) consisting of a special toothbrush, stabilizer solution and empty tubes. All tubes were already labelled with a primary subject code (PSC) consisting of 7 numbers (the first two numbers defining the MARK-AGE recruiter centre followed by a five digit number). In order to alleviate sample processing in the recruiting centres, cryotubes were coloured according to the destined sample type (red: EDTA

samples, blue: lithium-heparine samples, white: serum samples, yellow: urine samples, green: BMCs and whole blood samples).

2.5. Blood collection

All subjects were asked to donate blood (50 ml) by phlebotomy after overnight fasting. EDTA, lithium heparin and serum monovettes were used. One EDTA monovette containing 2.7 ml whole blood was sent to the local clinical chemistry laboratory for blood counts. Haemoglobin (Hb), hematocrit (Hct), erythrocytes, mean cell volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocytes, thrombocytes, eosinophil granulocytes, basophil granulocytes, neutrophil granulocytes, lymphocytes and monocytes were assessed. All other monovettes were processed to obtain plasma, serum, and peripheral blood mononuclear cells (PBMC).

2.6. Buccal mucosa cells collection

Prior to harvesting, volunteers had to rinse their mouth thoroughly with tap water to clear food residue from the oral cavity. Buccal mucosa cells were harvested by brushing each cheek 25 times from upside down with medium pressure using a special toothbrush (TEPE special care, TePe Mundhygieneprodukte Vertriebs GmbH, Hamburg, Germany). The cells were collected by washing out the toothbrush in a 50 ml test tube containing an aliquot of 4,5 ml stabilizer solution. If the cell solution was not turbid enough the brushing of cheeks was repeated (10 times each cheek) using a new toothbrush and rinsed in the same test tube again. Afterwards the entire stabilizer-BMC suspension was pipetted into a 5 ml cryotube and frozen at −80 °C until shipment to the Biobank.

2.7. Urine collection

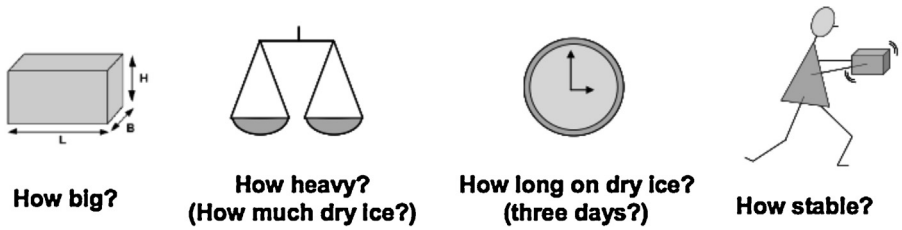
The urine collection took also place at the recruiting centres. MARK-AGE subjects were asked to collect at least 19 ml urine in a sterile screw-top container. The obtained volume was split in 14 ml urine + 140 μl 0.1 M Sodium azide (NaN_3) as preservative and 5 ml just urine following the requirements of analytic laboratories.

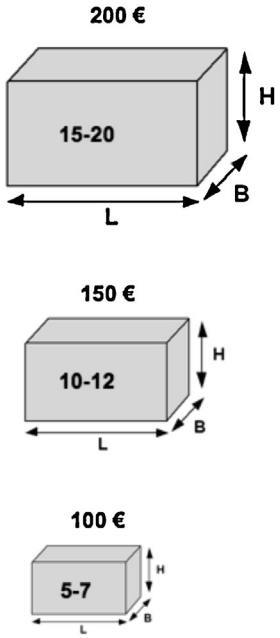
2.8. Database

The MARK-AGE database served as a 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. In a test phase the procedure of entering data in the database was "rehearsed" repeatedly by the Beneficiaries, in order to prevent any problems during the phase of active recruitment. The establishment of the database is described in detail in a separate manuscript (see Moreno-Villanueva and co-workers, this issue).

2.9. Samples shipment

Aliquots of biological material were stored at −80 °C (BMC, whole blood, serum, plasma and urine) or at -196 °C liquid nitrogen (PBMCs) at the recruitment centres until their shipment to the Biobank. At the beginning of the project size of the packets and the amount of dry ice necessary for keeping samples frozen during 3 days had been estimated. Also a fictitious shipment, including





to Hohenheim from	shipment/ month	samples/ packet	weight	aprox. size (cm)	aprox. volumen (litre)
Belgium	1	15	15	50x32x32	50
Austria	2	15-20	15-20	50x32x32 55x32x32	50-56
Greece	2	6-8	5-6	33x24x24	20
Poland	1	15	15	50x32x32	50
Italy	1	24	20	55x32x32	56
Netherlands	1-2	15-30	15-25	50x32x32 55x32x32	50-56
Finland	1-2	15-30	15-25	50x32x32 55x32x32	50-56
Biotesys	1	15-20	15-20	50x32x32 55x32x32	50-56

Fig. 1. Samples shipment from recruiting centres to the Biobank (Hohenheim, Germany).

shaking the packets in regular time intervals (as they would be moved by means of transport) had been performed at the coordinator centre. All samples from one proband were packed into a PSC-labelled plastic bag. Packets containing several bags were shipped on dry ice to the Biobank to a stipulated time point in a regular manner (Fig. 1).

At the Biobank samples were further aliquoted, re-labelled and transferred to the corresponding storage devices. They remained temporary stored until their shipment to the analyzing centres. Just before shipment tubes were transferred from storage devices into laboratory-specific cryo-safe storage boxes (form and size differed between analysing centres). These boxes containing the tubes were shipped on dry ice to the analytic laboratories in regular intervals depending on their store capacity. The analysing centres stored the boxes in the corresponding storage devices until analysis were performed.

3. Results

3.1. Recruitment strategy

The principal strategy was to get the attention of the population using the communication media, several articles and interviews in local and national newspapers and TV programmes. Recruitment centres gave an extended report on the MARK-AGE project and encouraged the population to participate leaving a contact telephone number. The recruitment strategies and MARK-AGE populations are described in detail in a separate manuscript (see Capri and co-workers, this issue). A great effort was also given in the recruitment of DS individuals and their families. The first meeting with them was at home in order to get acquainted with volunteers, to get the informed consent, and to answer the first part of the questionnaire together with their parents or care givers. The second part of the questionnaire with the specific battery of tests was performed with a second appointment (sometimes also a third) to complete all the interviews.

3.2. SOPs for sample collection and preparation at the recruiting centres

The first important element of the MARK-AGE SOP protocol was the usage of identical material for sample collection, preparation and storage. As described earlier recruiting centres were supplied with the same sample collection material (Sarstedt, Germany) and cryo tubes for sample storage (Greiner bio-one, Germany) by the Biobank. Due to the extremely sensitivity to freeze-thawing cycles, PBMCs and whole blood samples were already split for the analytic centres at the recruitment centres and only re-labelled at the Biobank.

All recruitment centres ordered the same reagents and material for PBMC isolation: Percoll (Amersham Biosciences), RPMI (Invitrogen/Gibco), DMSO (Sigma Aldrich, Germany, Penicillin/Streptomycin (Invitrogen, Germany), sterile sodium azide (NaN_3) (Merck, Germany). BioTeSys provided a stabilizer solution for BMCs. In order to avoid variability between different foetal calf serum (FCS) charges, the same FCS charge was used for all samples (Merck Millipore, Germany). Phosphate buffered saline (PBS) was ordered separately in each recruiting centre.

All biological samples were processed within 2 h and 10 min following a master protocol elaborated for MARK-AGE purposes (Fig. 2). By the use of two centrifuges (1× at room temperature, 1× at 4 °C) the different preparation tasks were able to manage in parallel in order to shorten the total time until freezing to a minimum. The collected urine was split by at the recruiting centre into two aliquots of 5 ml and 14 ml urine as indicated in chapter 2.3. Urine aliquots were frozen at −80 °C until shipment to the Biobank. In summary, whole blood, serum and EDTA/LiHep plasma aliquots were prepared from the blood monovettes, and stored at −80 °C immediately after cryotubes were filled. Further, PBMCs were isolated by density gradient centrifugation using Percoll (70% Percoll (GE Healthcare, Germany) + 10% 1.5 M NaCl) and stored overnight at −80 °C in freezing containers (Mr. Frosty, Nalgene®, VWR, Germany) with freezing medium containing 20% RPMI 1640 (Thermo Fisher Scientific, Germany) (with 1% Penicillin/Streptomycin) + 70% FCS (Merck Millipore, Germany) + 10% DMSO (Sigma, Germany). The next day PBMCs were transferred to liquid nitrogen (−196 °C) until shipment to the Biobank. For each proband, sample collection time, sample preparation starting time and sample freezing time were documented by the recruiting staff. Abnormalities like erythrocyte contamination in any samples were also recorded.

In order to train the MARK-AGE teams involved in recruitment, sample collection and preparation, data collection and entry a workshop about standard operating procedures was organized. Afterwards the whole procedure of recruitment, samples collection and data entering was mimicked in a test phase before the real recruitment started.

3.3. Biobank

The aliquots obtained at the recruiting centres were regularly shipped on dry ice to the central Biobank for storage and distribution to the Beneficiaries who performed the respective analyses. A shipment plan was made in order to define the optimal packet size, volume and weight for the shipments to and from the Biobank. Packets from recruiting centres with samples from a maximum of 40 MARK-AGE probands each (about 1160 cryotubes) were accepted by the Biobank per week. Upon requirement of the analyzing centres batches of samples were retrieved from the storage and sent out for analysis. This routine was performed via an individual agreement with each Beneficiary depending of analytical requirements, e.g. peripheral storage capacity, analysed numbers etc. On average the Biobank organized 4 shipments consisting of 4–12 packets each per year. In other words every 2–3 months the Biobank supplied analytic partners with packets containing samples of about 100–300 probands.

Urine, plasma and whole blood samples were split into several smaller aliquots at the Biobank and then re-labelled with the secondary subject code (SSC) by the Biobank staff team. Afterwards, all 58 aliquots of each proband were stored in the corresponding freezers or tanks until shipment to the analytic centres. However, before all samples were distributed within the Consortium one monovette of the lithium-heparine plasma was sent for hepatitis testing to Beneficiary #14 (RIVM). The positive samples were not distributed to the Beneficiaries but eliminated after sterilisation. In summary, based on 3169 classified as successfully recruited volunteers in the study (see Capri and Moreno-Villanueva et al., this issue), more than 158,400 samples were re-labelled and temporarily stored at the Biobank (Fig. 3).

As indicated above all tubes provided by the Biobank were labelled with a primary subject code (PSC). Due to the risk of samples losing their labelling during shipment or handlings special heavy-duty labels (CILS, Worthing, UK) were used for coding samples.

3.4. SOPs for analytic centres

In the analytic centres PBMC cryo vials were removed from liquid nitrogen to a 37 °C water bath. A volume of 0.5 ml 37 °C warm thawing medium (90% RPMI + 10%FCS) was added drop wise. After one minute cell suspension was transferred into a polypropylene

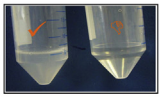
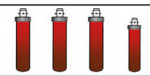
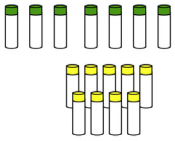
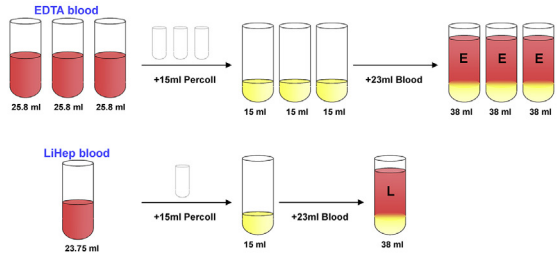
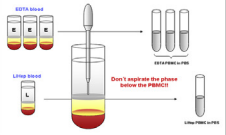
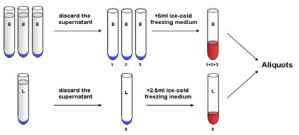
centrifuge 1 (RT)	centrifuge 2 (4°C)	tasks (parallel)	time (min)	total time elapsed
		Send one 2.7ml EDTA-monovette to your local clinical chemistry laboratory for blood counts		
	 	Freezing of buccal mucosa cells - transfer the entire stabilizer-BMC solution into a 5 ml cryo tube (green). Visually check: filling level should not exceed 4,5 ml (expected 4,3ml) - freeze 5 ml cryo tubes with cell suspension at -80°C in upright position to avoid accidental opening of caps - document how much blood has been detected within the stabilizer-BMC-solution	3	0h03min
		processing of EDTA- and LiHep-blood: You need 2x 50ml Falcons: - fill blood of the 3 EDTA-monovettes into 1x 50ml Falcon - fill blood of the 1 LiHep monovette into 1x 50ml Falcon	5	0h08min
centrifugation of EDTA- and LiHep-Falcons without brake: 300g, 15min (+x), RT (22-25°C)		prepare whole-blood aliquots for freezing from one 2.7ml EDTA-monovette: - **2x 1 ml cryo tube (green): 1000 µl** - **1x 0.5 ml cryo tube (green): 250 µl** - **1x 0.5 ml cryo tube (green): rest** urine aliquots for freezing: - mix the urine well before taking aliquots - **5ml cryo tube (yellow): 5ml urine** - **15ml Falcon (yellow): 14 ml urine+140 µl 0.1M NaN_3**	20	0h28min
		You need 2x 15ml Falcons: - remove EDTA- and LiHep-plasma and transfer each in a new 15ml Falcon (do not mix the EDTA- with the LiHep-plasma)	3	0h31 min
	centrifugation of EDTA- and LiHep-plasma (2x15ml Falcon): 2000g, 10min		11	0h42min
	centrifugation of 2 serum-monovettes: 2000g, 10min	prepare plasma aliquots for freezing: - **0.5ml cryo (blue): fill with 250µl LiHep-Plasma** - **5ml cryo tube (blue): fill with LiHep-Plasma** - **3x 5ml cryo tubes (red): fill with EDTA-plasma** - document if blood has been detected within the plasma samples	11	0h53min
		prepare Percoll-centrifugation: **Do not mix the EDTA- with the LiHep-blood cells!!** **You need 6x 50ml Falcons!** EDTA-cells: - dilute EDTA-cells (about 13 ml) with 25ml PBS - resuspend gently and distribute two times a third on two new 50ml Falcons - add to all three Falcons about 13ml more PBS and resuspend gently (you should have about 22 ml in each 50ml Falcon) - ratio cells:PBS should be approx. 1:6 LiHep-cells: - dilute LiHep-cells (about 3.5ml) with 21ml PBS and resuspend gently - ratio cells:PBS should be approx. 1:6 Percoll: - prepare three 50ml Falcons with 15ml Percoll-Solution for EDTA cells and one 50ml Falcon with 15ml Percoll-Solution for LiHep cells - transfer the EDTA-cells-PBS-dilution of each Falcon on top of 15ml Percoll - transfer the LiHep-cells-PBS-dilution on top of 15ml Percoll - tare falcons for percoll centrifugation **exactly!** - tare falcons adding PBS (not taking out blood)	20	1h13min
Percoll-centrifugation (4x50ml Falcon) without brake: 800g, 15min (+X), RT (22-25°C)		prepare serum aliquots: **2x 4 ml cryo tubes (white): fill with serum**	20	1h33min
		isolation of cells: **You need 4x 15ml Falcons:** - transfer EDTA-PBMCs of all 3x 50ml Falcons and collect them in three new 15ml Falcons - isolate LiHep-PBMCs of 1x 50ml Falcons and collect them in a 15ml Falcon washing of PBMCs: dilute PBMCs in 15ml Falcons with PBS (1+1 or more)	10	1h43min
	centrifugation (4x15ml Falcon) of washed PBMCs: 200g, 15min, 4°C		17	2h00min
prepare PBMC-aliquots for freezing: EDTA-PBMCs: - discard supernatant and add 5ml ice-cold freezing medium to the pellet of EDTA-PBMC - fill PBMC-solution into 12x 1ml cryo tubes **(red): 1x 1 ml, 5x 0.5ml, 6x 0.25ml (resuspend cells with pipette gently every time before you fill a cryo tube!)** LiHep-PBMCs: - discard supernatant and add 2.5ml ice-cold freezing medium to the pellet of LiHep-PBMC - fill PBMC-solution into 3x 1ml cryo tubes **(blue): 2x 1 ml, 1x 0.5 ml (resuspend cells with pipette gently every time before you fill a cryo tube!)** Keep the cryo tubes in a freezing container (Mr Frosty) in -80°C overnight and transfer next day to liquid nitrogen (-196°C)			10	2h10min

Fig. 2. Laboratory procedures for preparation of biological material. Elaboration of a very detailed master protocol for obtaining serum, plasma, whole blood and PBMC from the limited amount of blood (50 ml) to be taken from MARK-AGE subjects.

15 ml tube and thawing medium was added stepwise (1.0 ml, wait one minute, 2.0 ml, wait one minute, 4.0 ml, wait one minute). After centrifugation (250 × *g* for 10 min) cells were resuspended in RPMI cell culture medium or buffer according with the corresponding analytic procedures.

4. Discussion

Appropriate and optimised SOPs are the best basement of the success of a project especially when a large volunteers' recruitment is foreseen as well as MARK-AGE. Many beneficiaries were previously involved in EU GEHA project (Franceschi et al., 2007) thus having expertise in SOPs tasks including recruitment, samples collection and Biobank set up.

4.1. Recruitment

The preparation and launch of recruitment activity encountered some difficulties related to logistics. In some cases the interviews and blood draw needed to take place at the subjects' domiciles,

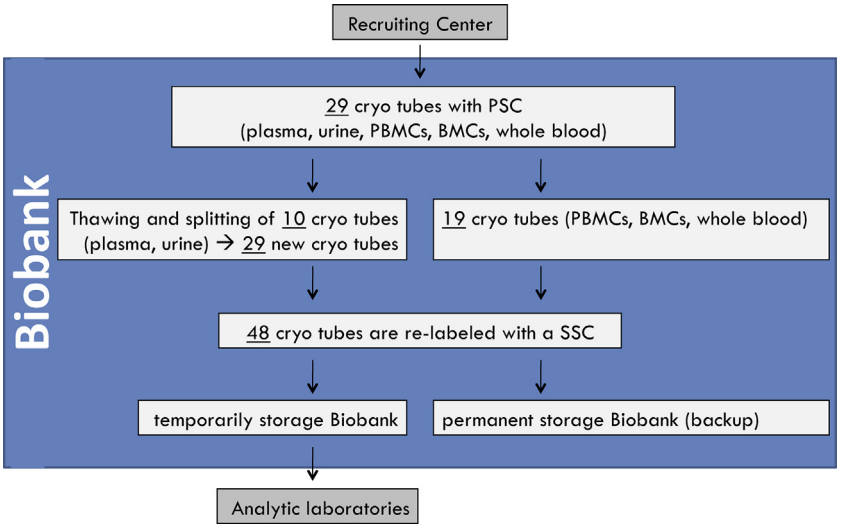


Fig. 3. Sample flow on one MARK-AGE subject.

especially in case of GO and SGO subjects. Unfortunately several of them were living in remote places therefore the time needed to process biological samples was critical for some of the intended biomarkers. Especially biomarkers related to oxidative stress could be affected due to the instability of those molecules. The influence of the transport time on biological material could not be checked for all biomarkers. Therefore, the Consortium decided to document the time of sampling collection, sample processing, sample storage and sample shipment in order to exclude those measurements influenced by time for logistic reasons.

Another tricky task regarded ethical issues, which had to be in agreement with current EU directives (Seppet et al., 2011). The elaboration of all necessary documents in a standardised manner appeared to be complicated due to differences in countries legislations. The Greek Ethic Committee did not allow taking more than 50 ml blood from old volunteers. This limited the amount of serum, plasma, PBMC and whole blood and forced the Consortium to elaborate a protocol, which maximal exploits the available biological material.

4.2. Samples collection

Right at the onset of the recruitment activity, extensive quality control analyses were carried out by Beneficiary #1 during the test phase in order to ensure the quality of the biological material coming from the recruitment centres. The analyses were focused on the quality of PBMCs and included cell counts, DNA damage and repair measurements and analyses of cell death (apoptosis and necrosis). The data showed that in many cases the number of viable cells obtained was inacceptable. Consequently the cryopreservation and thawing procedures for PBMCs, samples transport on dry ice and sample labelling at the Biobank were optimised. Afterwards the viability of the PBMC was improved from 30% to 80–90%. Beneficiary #21 repeated the analyses and the results were confirmed.

Another challenge was to provide the analytic laboratories with sufficient biological material for running their measurements. This was especially the case for PBMC aliquots, unfortunately a high percentage of the samples did not contain the necessary amount of cells. Therefore the sample distribution was rearranged and the affected Beneficiaries could be provided with additional PBMC aliquots. In addition, Beneficiaries also invested time and resources in the optimization of their assays in order to make them more sensitive and to be able to detect their potential biomarkers in lower amounts of material.

These optimizations were carried out using samples from additional volunteers (not enrolled in the MARK-AGE population) and which were, in part, provided by Beneficiary #1.

The effect of temperature on biological samples is known (Pasella et al., 2013) therefore the quality of serum and plasma samples was monitored. Beneficiary #14 checked the incidence of hemolytic, icteric or lipemic samples since in such samples a meaningful analysis of clinical chemistry parameters may not be possible. The incidence reported was below 0.7%, thus attesting to the very high quality standards of the MARK-AGE samples.

4.3. Biobank

One of the problems for standardised sample collection was the difference in existing laboratory material at the recruitment centres. In order to equip all recruiters with standardised material the Biobank ordered those from one central source. Another problem, relatively unique for the MARK-AGE Biobank, is the requirement of storage of different samples (blood, cells, plasma, serum, urine) in different volumes. In contrast to other Biobanks, e.g. storing only DNA samples, each of the samples has its special requirements, as tube size and storage condition. Whilst there is no perfect solution, processing and storage of biological samples is done as a compromise and within the constraints of a definite budget. A limited number of samples remain in the Biobank, for future usage by the Consortium.

5. Conclusions

By careful preparing the SOPs the MARK-AGE Consortium was able to handle the samples from 3169 probands. Standardization of blood collection material and procedure allowed a maximal comparison of samples from different recruitment centres and allowed also a maximal security of sample handling. The careful monitoring of sample flow by the Biobank and the exchange of subject codes by the central database minimized sample loss, ensured sample quality and guaranteed the double-blind design of the study. In general it can be concluded that restricting SOPs are required to ensure quality of a large cohort trial, especially if various metabolic samples are part of the study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2015.03.007.

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