Part B

Cover page

Proposal full title: Impact of Prenatal Stress on BRAIN AGEing

Proposal acronym: BRAINAGE

Type of funding scheme: Collaborative Project (small or medium-scale focused research project)

Work programme topics addressed: HEALTH.2011.2.2.2-2: Linking human development and ageing

Name of coordinating person: Prof. Dr. Matthias Schwab, MD PhD

List of Participants

Participant no.	Participant legal name	Country	Organisation type*
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2	Leibniz Institute for Age Research - Fritz Lipmann Institute, Jena (Dr. M. Platzer, Dr. J.P. Tuckermann)	Germany	Research Institution
3	Academic Medical Center Amsterdam (Prof. T.J. Roseboom)	Netherlands	University
4	Catholic University Leuven (Prof. Dr. B.R.H. van den Bergh)	Belgium	University
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1.1 Concept and objectives

Healthy mental ageing is one of the most prominent human wishes. Quality of life in aged subjects is mainly determined by the biological, not the numerical brain age. Better understanding of processes that cause early brain ageing predisposing to brain diseases will allow the development of diagnosis and early interventional strategies that lengthen the individual healthy life span and minimizes the time of frailty and dependence. Both genetic and epigenetic effects together shape phenotype and susceptibility to stress sensitivity and dysfunction of the cerebrovascular system, and decline of brain function during later life.

Stress sensitivity depends on the activity of the autonomous nervous system (ANS) and the hypothalamo-pituitary adrenal axis (HPA) axis. Hyperactivity of the HPA axis leads to central and peripheral GR resistance. Stress sensitivity contributes to biological ageing not only through excessive catecholamine and glucocorticoid (GC) secretion but also through central and peripheral GC receptor (GR) resistance. The latter increases the production of pro-inflammatory cytokines, accentuating potential neuronal damage. As stress is increasingly prevalent in society, a stress sensitive brain is particularly vulnerable to an early loss of brain resilience to challenges. Stress sensitivity is programmed prenatally mainly by prenatal stress, GC exposure and undernutrition. Moderate undernutrition during pregnancy is widespread in both developing countries and western societies like the EU. In industrialized countries, maternal lifestyle in which dieting (including global food reduction) is used for cosmetic reasons, is a common cause of moderate undernutrition. A recent study showed that most women do not improve their dietary and lifestyle patterns in pregnancy.[1] Poor fetal nutrition also occurs in teenage and elderly primigravid pregnancies.

Brain ageing is also related inseparably to cerebrovascular function. The incidence of stroke – the most common brain disease – increases with age when the functionality of the vascular system decreases and additional risk factors e.g. arteriosclerosis appear. Stroke is the third most common cause for disability and invalidism in Europe. Costs for prevention, treatment, rehabilitation and care are 4% of the entire health budget in Germany and is increasing with higher life expectancy.

We hypothesize: (1) prenatal stress and undernutrition program premature brain ageing; **(2)** premature brain ageing is due to epigenetic changes that decrease GR sensitivity, alter activity of the ANS and increase cerebrovascular tone; and **(3)** premature brain ageing predisposes to age-associated diseases including cognitive decline and stroke.

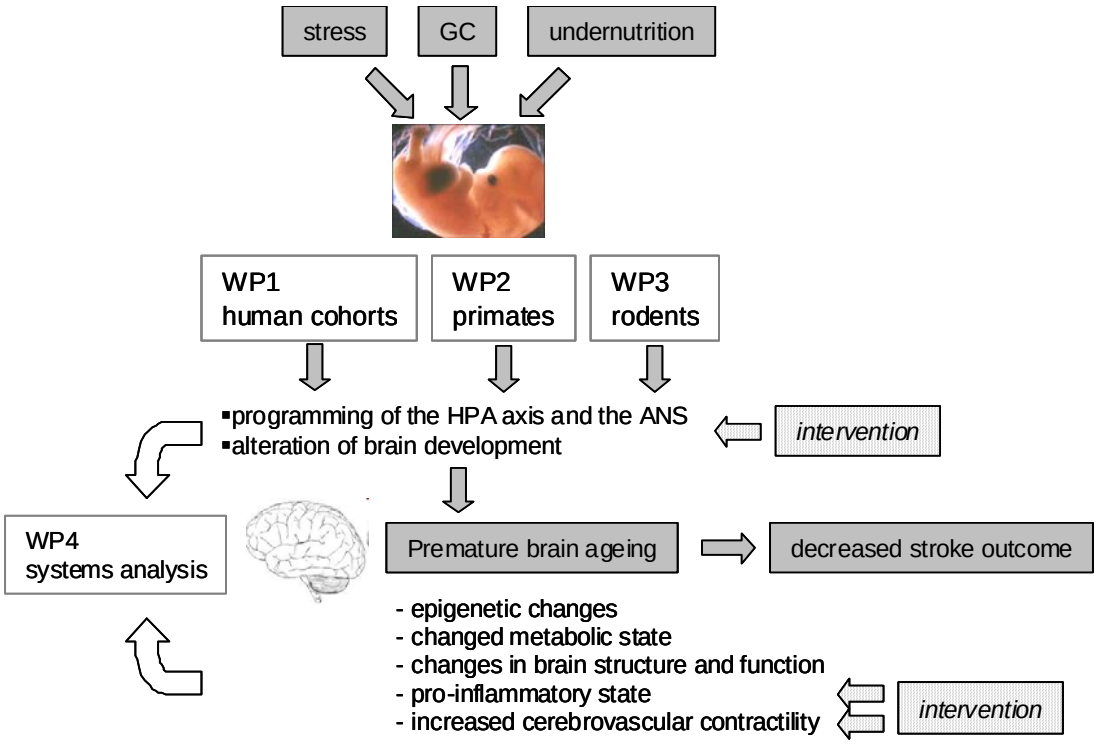

Fig. 1. Study concept.

[1] Crozier et al., *J Nutr* 2009 **139**:1956-63.

Objectives: The consortium will establish strategies for the early diagnosis of brain ageing. We will develop treatment strategies to aid healthy brain ageing and reduce the burden of age-associated brain disorders. In an integrated and translational approach, we will **(I)** determine the effect of major environmental factors (prenatal stress, GC exposure and malnutrition), that may induce epigenetic modifications, on brain development and brain ageing in humans using innovative routine parameters and standardized approaches in order to overcome the low reproducibility of results in existing studies. This approach is indispensable for a clinican's ability to dissect effects of different epigenetic processes that emerge phenotypically noticeable at various ages. The corresponding use of non-human primates gives us a truly unique opportunity to translate experimental results from rodents to humans.
We will **(II)** analyze in experimental studies to what extent GC resistance, increased sympathetic activity or increased cerebrovascular tone are key mediators connecting prenatal epigenetic modifications to premature brain ageing. Based on preliminary studies we expect that these epigenetic modifications increase the susceptibility of the aged brain to stroke. We will **(III)** show in a human cohort that stroke outcome is worse in the prematurely aged brain. The underlying mechanisms will be dissected in experimental studies. We will finally **(IV)** identify pharmacological interventions that may reverse altered stress sensitivity and vasoreactivity in rodents as a base for future preventive or therapeutic human studies.

1.2 Progress beyond the state–of-the-art[2]

It is a well established that epigenetic modifications mediated by prenatal stress and GC exposure have a significant impact on health for the entire duration of an individual's life. The current (true but very simplified) concept is that stress sensitivity is programmed prenatally in the last third of gestation when the HPA axis matures. If GC concentrations are high at this time, GR expression and sensitivity in the hippocampus and hypothalamus, which is important for negative feedback to "turn off" the stress response, is permanently reduced by epigenetic modification of the GR genes. This mechanism results in a hyperdrive of the HPA axis in the presence of GR resistance. However, clinical and experimental studies have shown that prenatal stress early during gestation, i.e. before the HPA axis matures, also alters stress sensitivity. Our data indicate that this is due to altered development of the ANS.[3] Apart from the effects of prenatal stress and GC exposure on the stress system, they are also highly likely to affect brain development, with persistent effects.[4] Even moderate maternal undernutrition affects development of neuronal network formation, as we have shown in baboon pregnancy.[5] Low nutrient supply also stresses the fetus, thus programming its developing stress system.[3] The first direct evidence in humans that prenatal undernutrition affects later health has come from studies showing that people exposed to the Dutch famine prenatally have an increased prevalence for chronic diseases that plague our society, such as type 2 diabetes, cardiovascular disease, or increased stress responsiveness.[6] Recent evidence suggests that cognitive function may also deteriorate faster in those subjects who were prenatally exposed to the famine.[7] Similarly, our own studies in human cohorts (with ages up to 20y) suggest that prenatal stress and GC exposure affects activity of the ANS and HPA axis resulting in higher cortical dysfunction, behavioural problems and increased probability for depression.[8-11]
Effects of prenatal stress and GC exposure on stress sensitivity during later life seem to depend on poorly determined conditions. Following exposure, human and animal studies show that the HPA axis and the ANS are even less active during certain stages of life. These data are inconsistent probably because the "programming effects" depend on a complex mixture of environmental factors, the time and the length of exposure. Complicating, different methodical approaches were used to detect the altered activity of the stress system. In addition, GR resistance may also exist in the absence of a measurable enhanced stress response. Often, it is simply difficult to measure the activity of the ANS. We have developed innovative nonlinear heart rate variability (HRV) parameters[11] that reliably reflect the disturbed ANS development after prenatal

[2] **All subsequent citations concern work of the participants of the consortium. References to the work of other groups will be given in the full proposal.**
[3] Frasch et al., *J Physiol* 2007 **579**:893-907
[4] Antonow-Schlorke et al., *Obstet Gynecol* 2009 **113**:142-51
[5] Antonow-Schlorke et al., *PNAS* in press
[6] Roseboom et al., *Heart* **2000** 84:595-8
[7] De Rooij et al., *PNAS 2010* **107**:16881-6
[8] Van den Bergh et al., *Neurosci Biobehav Rev* 2005 **29**: 237-58
[9] Mennes et al., *Clin Neurophysiol* 2009 **120**:1116-22
[10] Van den Bergh et al., *Neuropsychopharmacol* 2008 **33**:536-45
[11] Frasch et al., *Am J Physiol* 2009 **296**:R702-7

stress or maternal undernutrition.[3] To understand effects of different environmental factors, that may induce epigenetic modifications, on brain function and ageing, systematic and contemporaneous studies of clinical and experimental factors should use the same methodical approaches as proposed in this application.

Our preliminary experimental results suggest that prenatal GC exposure induces altered stress sensitivity in aged rats associated with accelerated brain ageing including depressive behaviour and cerebrovascular dysfunction (Fig. 2). While the interaction of the stress and the serotonergic system may explain the occurrence of depressive disorders, there is no precise understanding of how increased stress sensitivity induces cognitive disturbances. Complex indirect effects may play a role. For example, resistance of peripheral GR to the immunosuppressive GC leads to a pro-inflammatory state that has negative effects on neuronal function. In agreement with, our preliminary results show that a pro-inflammatory state occurs already in the normally aged rodent.

Cerebrovascular dysfunction and an insufficient suppression of secondary cerebral inflammation after prenatal GC exposure are associated with larger stroke volume and decreased stroke outcome, particularly in older animals (Fig. 2). A considerable amount of neurons fade away during the secondary cerebral inflammation following stroke. The profound physical and psychological stress after ischemic stroke activates the sympathetic nervous system and the HPA axis that inhibit pro-inflammatory cytokine production. Own preliminary experimental data suggest that inflammation is enhanced following prenatal GC exposure due to GR resistance in the immune system (Fig. 2). In agreement with this, mice with GR deficiency in the immune system (GR ^{LysMCre}) also show increased proinflammatory activity and decreased stroke outcome. The latter is possibly also related to decreased neuronal plasticity limiting functional recovery. Treatment with SSRI reverses the increased vasotone (Fig. 2) and, probably, GR resistance and the pro-inflammatory state.

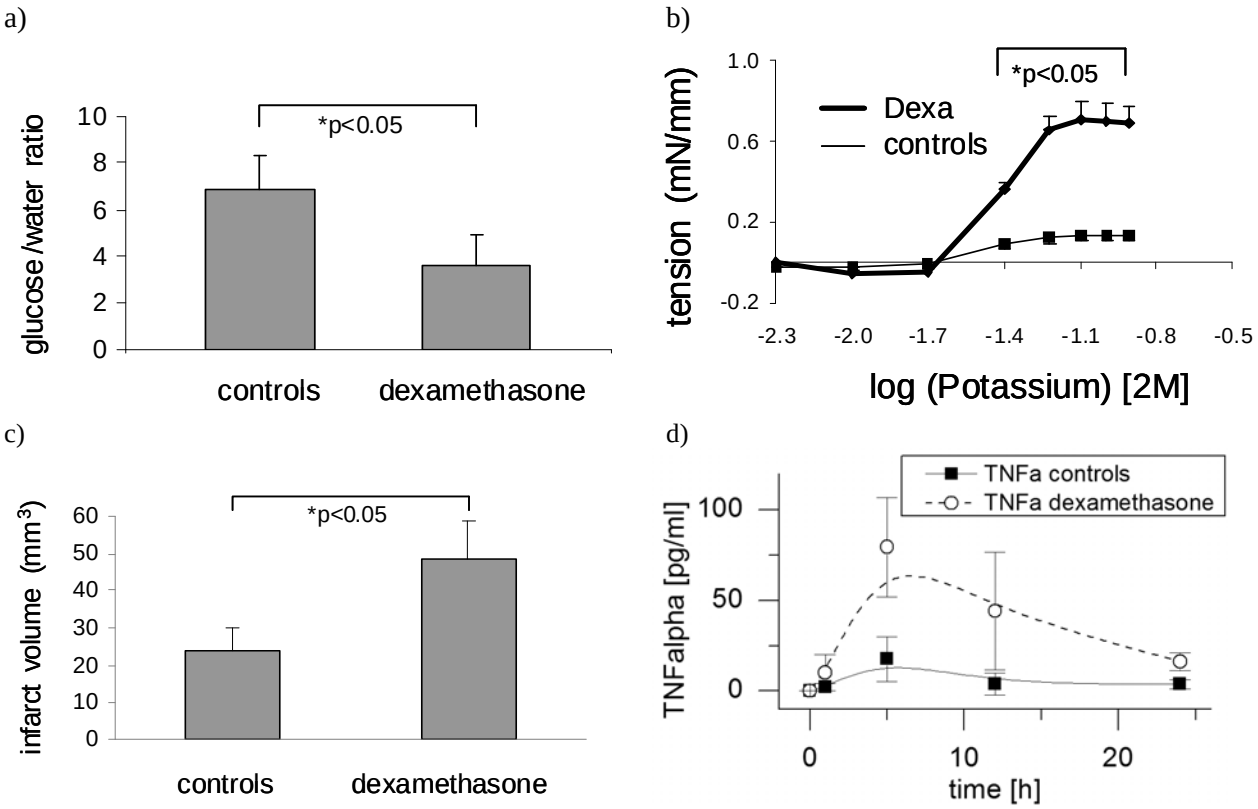

Fig. 2. Prenatal dexamethasone exposure in rats at day E19 and 20 at the dose used clinically to enhance fetal lung maturation in threatened premature labor (a) induces anhedonia (glucose water test); (b) increases cerebrovascular tone; (c) increases release of pro-inflammatory cytokines to an infection and, consequently; (d) induces larger brain infarctions at the age of 2y.

1.3 S/T methodology and associated work plan

The work plan is scheduled for 5y because of the need to examine several human cohorts and to follow-up rat and mice, after prenatal intervention, until 2.0y of age.

Work package 1: human cohort studies

Aims: 1. to examine the effects of major environmental factors (prenatal stress, GC exposure and undernutrition) on brain development and brain ageing.
2. to show that the stroke outcome is decreased in the prematurely aged brain and associated to an altered stress sensitivity.

The specific expertise of our groups in examining environmentally induced disturbances of the activity of the HPA axis and the ANS, externalized and internalized behaviour disorders, cognitive development and structural and functional changes in the MRI in human cohorts will be integrated to allow high level standardized examination of the cohorts. The cohorts with prenatal GC exposure were exposed to betamethasone (BM) to enhance fetal lung maturation but did not deliver prematurely. We use cohorts including controls. The following cohorts are available at the age of:

- **2-3y**: prenatal stress (cohort 1), BM (cohort 2) and undernutrition (cohort 3)
- **12 and 25y:** prenatal stress (cohort 4), BM (cohort 5) and undernutrition (cohort 6)
- **67y:** the Dutch famine cohort (cohort 7)
- **>50y:** stroke patients (cohort 8) recruited at the Dept. of Neurology, University hospital Jena (> 400 strokes/year)

We will examine

structural changes:	- MRI (MRI based volumetry)
functional schanges:	- cognitive function (non-linear EEG analysis, neuropsychology)
	- inflammatory state (cytokines)
	- metabolomics*
	- neuronal plasticity (motor learning) in the stroke cohort
genetic changes	- telomere length, next generation epigenetic sequencing**
cerebrovascular function:	- cerebral autoregulation (derived from spontaneous blood pressure and CBF fluctuations) in the Dutch famine cohort)

We will relate these parameters to indicators of stress sensitivity:
- activity of the HPA axis (cortisol at baseline and under a standardized stress paradigma: Trier stress test)
- degree of GR resistance (on immune cells using peripheral blood)
- activity of the ANS (HRV analysis, spontaneous baroreflex).

* Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind, specifically, the study of their small-molecule metabolite profiles. Thus, while mRNA gene expression data and proteomic analyses do not tell the whole story of what might be happening in a cell, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. Whereas biomarkers are available for the diagnosis of many diseases, e.g. acute myocardial infarction, no such markers exist for brain age or the diagnosis of stroke.

** Genome-wide profiles of DNA methylation and acetylation markers using Chip-seq of brain tissue and blood samples with acetylation antibodies on Illumina/Solexa GAIIx sequencers.

Work package 2: studies on non-human primate studies[12]

Aims: 1. to translate the experimental results in rodents (work package 3) into humans
2. to compare the effects of IUGR with those of moderate undernutrition

We will provide translational power with the animal model closest to the human and one in which the burden and frequency of investigation is more than possible in humans throughout their life span. The non-human primate deliverables will be the same as in the human studies for example we have developed the use of the Cambridge Neuropsychological Test Automated Battery used for human cognitive testing. We have available cohorts

- at the age of 6 to 7y equivalent to 24 and 28y in humans (cohorts 9 and 10) with prenatal moderate undernutrition in pregnancy and lactation (a situation in which fetal cortisol is spontaneously elevated) or maternal administration of BM at the levels used in humans clinically to enhance fetal lung maturation. The cohorts showed modest behaviour disorders at younger age. We will test the groups in the first two years and then again in years 4 and 5 when each group will be 3 years older, the equivalent of 35 to 40y.

[12] The cohorts will be co-financed by the NIH.

Work package 3: experimental studies in rodents and transgenic animals

Aims:
1. to examine to what extent GR resistance, increased sympathetic activity and cerebrovascular tone mediate between prenatal epigenetic modifications and premature brain ageing
2. to compare the impact of the environmental factors prenatal stress, GC exposure and undernutrition which have, potentially, epigenetic impact
3. to consider the age when epigenetic effects become phenotypically apparent
4. to dissect the mechanisms for the decreased stroke outcome (preliminary result, see state-of-the-art) following prenatal stress and GC exposure
5. to identify pharmaceuticals that reverse altered stress sensitivity or vasoreactivity

Work package		participant	task	year				
				1	2	3	4	5
1	**human studies**							
		1	manages cohort 5, 6, 8; cohort study					
		2	epigenetic and GR examinations					
		3	manages cohort 1,2 and 4, cohort study					
		4	manages cohort 7, cohort study					
		6	metabolomics					
		7	EEG analysis					
2	**studies on non-human primates**							
		1	EEG recordings					
		2	epigenetic and GR examinations					
		3	behavioral tests					
		4	cardiovascular tests					
		5	owns cohort 9 and 10, primate study					
		6	metabolomics					
		7	EEG analysis					
3	**experimental studies**							
		1	rodent experiments					
		2	epigenetic and GR examinations					
		6	metabolomics					
		7	EEG analysis					
4	**systems analysis**	all	integrated data analysis					

Tab. 1. Overview of the time line of the studies and the integrated action of the participants.

We will use the methods given in work package 1 adapted to the requirements of animal experiments (e.g. behavioral tests instead of neurpsychiatry) and add small vessel myography to test cerebrovascular reactivity. We will use both sexes. Stroke will be induced using a standard model (MCAO). Stroke volume, neuroinflammation and central and peripheral GR resistance will be estimated. To dissect the relevant pathways we will use

- mice with GR knocked out in neurons (exhibit increased ACTH and corticosterone production, $GR^{CamKIVCre}$), T cells (GR^{LckCre}), macrophages or microglia (the macrophages of the brain, $GR^{LysMCre}$)
- mice with knockouts of specific pathways of GR mediated transactivation / -repression of gene transcription (GR^{dim} and to be generated). Effects of activated GR on transactivation / -repression are tissue specific and depend on unknown circumstances. Examining these mechanisms is necessary to develop specific GR antagonists
- pre-treatment with RU486 (to block GR), spironolactone (to block mineralocorticoid receptors), SSRI (that immediately normalize GR resistance and pro-inflammatory state and decrease vascular reactivity independently of the delayed effects on serotonin re-uptake) and ß-blockers

Work package 4: Systems analysis

Aim: Integrated analysis and comparisons of all study groups with the aid of the "Jena Center for Systems Biology of Aging".

Milestones:

Milestone 1: At the end of the **first year**, all protocols will have been standardized and fixed. They will have been adapted to the different species.

Milestone 2: At the end of the **third year**, successful data sampling will be demonstrated for all cohorts; potential delays will be identified and care will be taken to ensure progress in the remaining time.

Milestone 3: At the end of the **fourth year**, all data except for some experimental data from mice will have been collected.

Milestone 4: At the end of the **fifth year**, comparative analysis of the data should be finished.

2. Impact

2.1. Expected impacts listed in the work programme

The increased incidence of brain diseases with longevity is not only a health problem, but also a social problem. There is considerable anxiety that its cost could cause a collapse of the social and health insurance systems, which already operate near their limits, in the forthcoming future in Europe. This situation requires information from scientific advances that can quickly be translated into prevention and therapy. We have chosen a strong translational approach to investigate the impact of epigenetic modifications on brain ageing. This will support the development of relevant early diagnostic, preventive and therapeutic approaches. The use of standardized approaches in different human and non-primate cohorts greatly enhances their translational value. It is obvious that starting prospective epidemiological studies on epigenetic programming of health and disease in later life will not generate results in the foreseeable future. This project offers the unique opportunity to use the well-defined available cohorts that are not available in any single nation in the world. Bundling of the state-of-the-art methods available in our consortium and the standardized examination of our cohorts will lead to new insights and set new standards in the clinical examination of epigenetic effects. This also will raise the quality of future follow-up examinations of our cohorts at a new level. Added value is also raised from the close interactions of scientists who focus on human development with those engaged in the field of ageing.

Our results will be of immediate relevance for the ageing society. We will experimentally test pharmacological, and possibly other preventive and interventional strategies which can rapidly be transferred to humans due to the translational focus project of the project. Our work may therefore contribute to the reduction of financial burden on national health care providers. As a first clinical relevant step, we will develop markers of premature brain ageing to select individuals vulnerable to brain disorders. In this context, the SME Biocrates will use knowledge generated during the project for the development of metabolic markers that are suitable to estimate biological brain age. The SME ANT will develop electrophysiological markers for brain ageing. The knowledge obtained in this project will facilitate ideas and advancement of our and other SMEs.

3. Consortium and Resources to be committed

Our well-balanced consortium consists of participants with great experience and focus on fetal development and ageing. The experience ranges from cell biology and genetics, whole animal physiology and phenotyping, to clinical studies in infants and elderly subjects. The cohorts, models, knowledge and methods which the participants contribute are complementary. Our methods will be standardized for all subjects and places. This creates a link between epigenetic modifications during human development and premature brain ageing. **Participant 1** consists of members of the Depts. of Obstetrics and Gynecology and Neurology at the University Hospital Jena. They combine expertise in experimental and clinical research in the fields of fetal programming, ageing and stroke.[13] The group has developed a model of prenatally programmed stress sensitive rat and mice that show increased cerebrovascular tone and cerebral inflammation following stroke. Innovative nonlinear EEG and HRV parameters were developed by the participant to quantify neuronal activity and measure the tone of the ANS. The group demonstrated the suitability of the HRV parameters as prognostic markers in critical illness.[14] Based on a large database of MRIs and on detailed volumetric processing algorithms they can estimate the biological brain age form MRI structure by less than +/- 5y. **Participant 2** comprises groups working on genome and epigenome analysis and tissue specific GR activity at the Leibniz Institute for Age Research. They have extensive experiences in gene sequence analysis in mouse, rat, and primates and functional genomic and DNA methylation and histone acetylation analyses aiming for longevity.[15-19] The participant also has comprehensive experiences in cell biological analysis of the pathways and tissue specific regulation of gene expression after binding of GC to the GR.[20-22] Participants 1 and 2 collaborate in the Jena Center for Systems Biology of Ageing (JenAGE).

Participant 3 is Associate Professor and Principal Investigator at the Dept. of Clinical Epidemiology, Biostatistics and Bioinformatics and the Dept. of Obstetrics and Gynaecology at the Amsterdam Medical Center. She has followed up the unique Dutch famine groups as a the only well-characterized model group of epigenetic programming at older age in the world. **Participant 4** is Full Professor for Biological Developmental Psychology and has outstanding experiences in behavioral and neuropsycological testings in the postpartum period and the follow-up of the offspring until adulthood. Her research group is focussed on the effects of prenatal stress on HPA-axis function and neurodevelopment involving physiological measures of maternal stress and of early sensory-cognitive development of the infant. She is also involved in public health research funded by the Flemish Government. **Participant 5** has a strong background in the endocrinology of the HPA axis and the mechanisms of epigenetic programming.[23] He is the director of the only Center in the world with a cohort of juvenile non-human primates with prenatal moderate undernutrition and an aged cohort that was exposed prenatally to GC. The cohorts will be co-financed by the NIH. An outstanding cooperation over 15y exists between Participants 1 and 5 leading to papers on the highest level.[2] Using metabolomics, **Participant 6** develops relevant biomarker of brain ageing and the diagnosis of stroke.[24]. **Participant 7** develops innovative EEG evaluation routines that will be used to develop electrophysiological markers for brain ageing. The consortium has access to all cohorts to be examined (see work plan). These cohorts exist and are – except of the Dutch famine cohort - prospectively characterized. The different models of tissue specific GR knockout mice are available (Participants #1 and #2).

The excellent infrastructure of the participating groups does not require applying for expensive equipment. The budget will be used for salary of postdocs and technicians. A considerable amount of money is needed to maintain the ageing animal colonies (rodents, baboons). Participant 1 has the largest need for human resources because the group performs human and experimental studies. The project management and yearly meetings will be crucial for the highly interactive work of the consortium. The budget for the other activities involves organization of two symposia to interchange ideas and share the results with the scientific community.

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[13] Schwab et al., *Lancet Neurol* 2008 **6**:102-4
[14] Schmidt et al., *Crit Care Med* 2008 **36**:967-70
[15] Watanabe et al., *Nature* 2004 **29**:382-8
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