PROJECT FINAL REPORT

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1. Executive summary

The increased incidence of age-related brain diseases with longevity poses not only health but also social and economic problems in today´s society. There is concern amongst political leaders and society in general that costs relating to the health and social problems of an increasingly ageing society could hugely challenge the European social and health insurance systems in the coming years. Hence innovative solutions which can quickly be translated into preventive and therapeutic strategies for early brain ageing and brain age-related disorders are urgently needed. Based on our own studies and on work by others, the *BrainAGE* consortium considers that prenatal stress influences brain health and disease in later life and analysis of this process is the key to identification of novel therapeutic procedures.

Our main hypothesis was that brain ageing is due to programming of an increased individual stress sensitivity following exposure to different exogenous and endogenous stressors during intrauterine life. Exposure of the fetus to increased maternal stress hormone levels is attributable to three main maternal factors: psychological stress, treatment with glucocorticoids and undernutrition.

BrainAGE aimed to identify the effects of these prenatal stress factors (maternal psychological stress, glucocorticoid exposure and malnutrition) on brain development and ageing as well as on age-associated brain disorders such as stroke and cognitive decline. We determined mechanisms linking early human development and ageing using molecular biological and genetic approaches and translated experimental results by using non-human primates into the human situation. We explored the efficacy of an early interventional approach based on lifestyle changes of the pregnant mother and a late pharmacological interventional approach in rodents as a base for interventional human studies. Preventing early brain ageing also has an impact on reducing brain-related diseases, such as stroke. Noticeably, *BrainAGE* strongly emphasized a gender specific examination of processes since stress sensitivity varies between sexes.

The results achieved in the project show **(I)** that prenatal stress programs structural and functional brain development and early cognitive decline as well as the outcome of stroke; **(II)** the extent to which the different types of prenatal stress program early brain ageing; **(III)** that epigenetic changes of growth hormone and glucocorticoid receptor genes increased stress sensitivity and metabolic changes are key mediators connecting developmental modifications to brain development and ageing; **(IV)** that a prenatally programmed increased cerebrovascular tone contributes to the decreased stroke outcome; and **(V)** positive effects of early and late interventional approaches. Finally, **(VI)** *BrainAGE* has developed and provides innovative MRI-based, neurocognitive and molecular biological strategies to detect early brain ageing by measuring the biological brain age compared to the chronological age.

Overall, we have obtained a better understanding of the impact of various prenatal stressors, and have achieved an enhanced detection of subtle changes in brain development and ageing. *BrainAGE* makes available new knowledge relating to the early environmental stimuli that should be avoided at particular times of human development. This knowledge will enable us to make recommendations to scientists, public health policy makers, and health professionals to empower and educate women to attempt to avoid adverse effects of prenatal stress hormone exposure. Our findings will enable policy makers to develop specific public health policies and increase public awareness with regard to ameliorating the ageing process and thus, allowing a better integration of an ageing community into society. In this context, we organised the first conference on the topic of prenatal stress and brain aging, bringing together leading scientists to discuss various aspects of the topic and to plan future directions of research for healthier brain aging. We have summarized the results from *BrainAge* in a special issue of the Journal “Neuroscience and Biobehavioural Reviews”. The dissemination activities, in particular to the general public on knowledge on vulnerable periods and adverse environmental stimuli during pregnancy will raise public awareness. Finally, our results can rapidly be transferred to relevant health policies at the

European and global level and will make significant contributions towards reducing disease burden and the financial burden on health care providers and health care systems.

2. Summary description of the project context and the main objectives

In view of the worldwide ageing population, understanding the biology of healthy ageing is more relevant than ever. In Europe, the progressively ageing population has prompted concerns amongst the inhabitants and politicians that a large fraction of the populace will suffer age-associated brain disorders such as stroke, cognitive decline and dementia. Stroke is the third most common cause for disability and invalidism in Europe. Costs for prevention, treatment, rehabilitation and care consume 4% of the entire health budget in Europe. This figure is expected to rise with increasing higher life expectancy. A major goal of the society is to find ways to ensure that this increase in longevity is also accompanied by an improvement in disease-free life expectancy. The question is how can therapy delay the onset of typical age-associated diseases? The prenatal period is increasingly recognized as a target for the primary prevention of diseases in later life.

The *BrainAGE* Project was a five-year EU FP7 funded project that brought together world-leading research expertise from the EU and the US to study the challenges for healthy brain ageing. We proposed that increased stress sensitivity which is programmed by exposure to stress during intrauterine life programs early brain ageing (Fig. 1). Exposure of the fetus to increased stress hormone levels is mainly attributable to three factors (related to the mother): maternal stress, maternal treatment with stress hormones and maternal undernutrition. Maternal stress is in part caused by the stress of modern-day life. Treatment with stress hormones is also very common in pregnant women. Almost 10% of all pregnant women threatening preterm delivery are treated with stress hormones (glucocorticoids) to enhance fetal lung maturation. Furthermore, moderate undernutrition during pregnancy, a cause of fetal malnutrition, is widespread both in developing countries and in Western societies such as the EU often due to a lifestyle comprising of a restricted dietary intake (including a global food reduction) for cosmetic reasons. A recent study showed that most women do not improve their dietary and lifestyle patterns during pregnancy. Finally, poor fetal nutrition due to relative placental insufficiency is also present in teenage and in elderly pregnancies, the latter being on the increase as more and more women choose to put off having children until their mid- or late thirties, or even their forties.

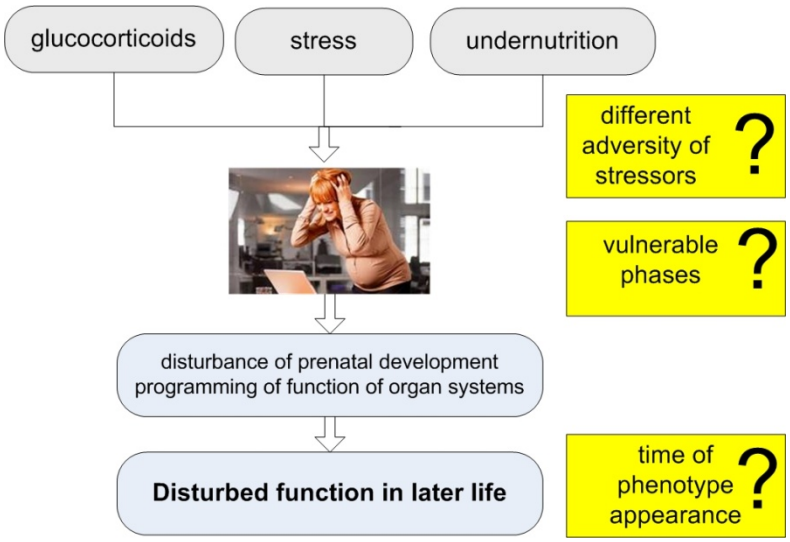

Fig. 1. *Effects of prenatal stress.*

Therefore, *BrainAGE* focused on the link between stress exposure during early human development, brain development and brain ageing and the most important age-related brain diseases: cognitive decline and stroke (Fig. 2). The relationship between the prenatal environment and brain ageing is undoubtedly complex involving a number of factors. To name some potential mechanisms: Exposure of the fetus to increased stress hormone levels leads to epigenetic reprogramming of central glucocorticoid receptor sensitivity and, thus to a decreased negative feedback control of cortisol release. This leads to enhanced cortisol release leading to increased stress sensitivity during the entire life span. A brain sensitive to stress is particularly vulnerable to an early loss of brain resilience toward challenges since cortisol has negative effects on neuronal

activity. Thus, increased stress sensitivity contributes to biological ageing and cognitive decline both through excessive stress hormone secretion and glucocorticoid receptor resistance. Moreover, increased stress hormone secretion increases vasotone and accentuates the production of pro-inflammatory cytokines, potentially accentuating neuronal damage after stroke.

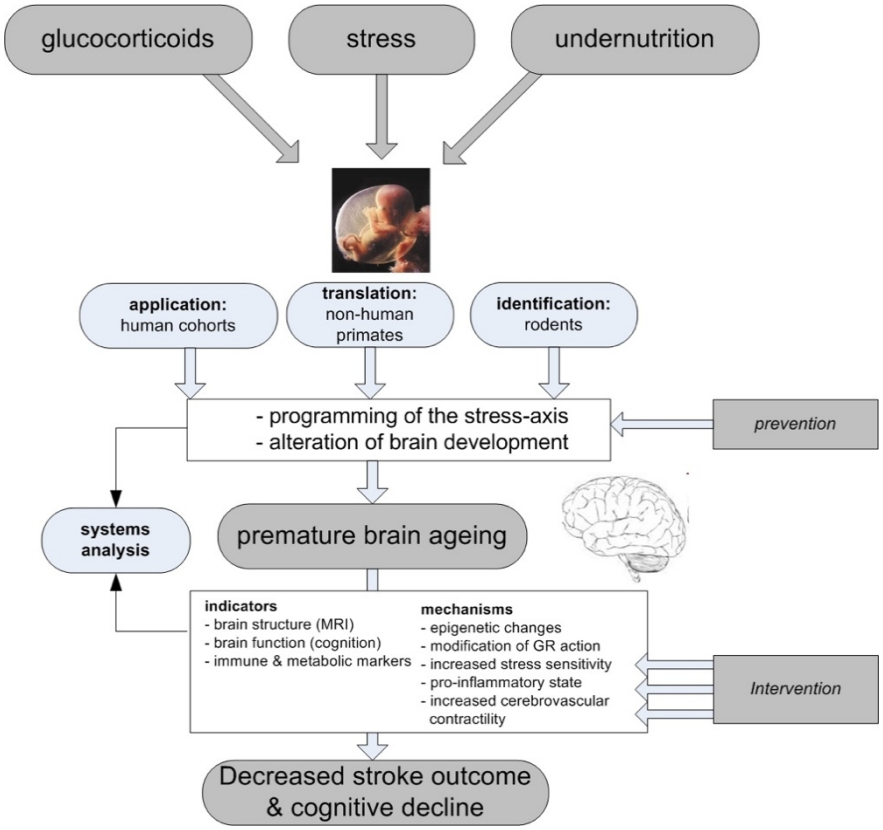

***Fig. 2**. Study concept*

Based on these considerations, *BrainAGE* examined the mechanisms underlying the link between prenatal stress and brain ageing and explored interventions which may be employed to support healthy brain ageing in subjects at risk. For this purpose, we compared the impact of different types of prenatal stress: maternal psychological stress, undernutrition, and glucocorticoid exposure. We developed indicators and measures for biological brain age in comparison to the chronological age. We explored early interventions when the condition is still reversible and late interventions which may be employed to support healthy brain ageing in subjects at risk.

We hypothesised: (1) prenatal stress programs early brain ageing; **(2)** early brain ageing predisposes to age-associated brain diseases including cognitive decline and stroke; **(3)** early brain ageing is due to epigenetic gene regulatory mechanisms that alter the activity of the hypothalamo-pituitary-adrenal axis and the autonomic nervous system, as well as due to changes in metabolism and immune function; **(4)** changes in immune function towards a proinflammatory state and an increase in cerebrovascular tone have negative effects on stroke outcome; and **(5)** interventions directed against increased stress sensitivity, pro-inflammatory state and increased cerebrovascular tone with serotonin re-uptake inhibitors reduce susceptibility to stroke in the stress sensitive brain.

Objectives: In an integrated and translational approach (Fig. 3), we **(I)** determined the effect of major prenatal stress factors (maternal psychological stress, glucocorticoid exposure and malnutrition) on brain development and ageing. We employed innovative techniques based on MR morphology and a theoretical foundation bridging behavioural and neurophysiological research in order to measure the biological brain age in a sensitive and standardized way overcoming the low reproducibility of results in existing studies. **(II)** We examined to what extent prenatal stress

programs early cognitive decline and incidence and outcome of stroke in aged subjects. **(III)** We analyzed to what extent epigenetic changes increased stress sensitivity, or induced changes in the immune state and metabolome which are key mediators connecting stress-induced developmental modifications to early brain ageing. **(IV)** We examined to what extent changes in immune function towards a proinflammatory state and an increase in cerebrovascular tone negatively affect stroke outcome. **(V)** We determined structural, functional and metabolic biomarkers for the risk of early brain aging. **(VI)** We tested the efficacy of an early interventional approach based on lifestyle changes of the pregnant mother and of a late pharmacological interventional approach with serotonin re-uptake inhibitors in rodents as a base for interventional human studies.

The results achieved in the project reveal to what extent the different types of prenatal stress program early brain ageing. They show the adversity of different stressors, disclose vulnerable phases to stress during pregnancy and examine the phenotypic appearance of the effects of prenatal stress.

The study of the prenatal environment in *BrainAGE* has very substantial public health implications in terms of improvement of the outcome for offspring because maternal life style and stress are modifiable. Therefore, the results of *BrainAGE* have the potential to considerably improve the health of future generations.

Overall approach: In designing the project and the work plan, we had to overcome several obstacles (Fig. 3):

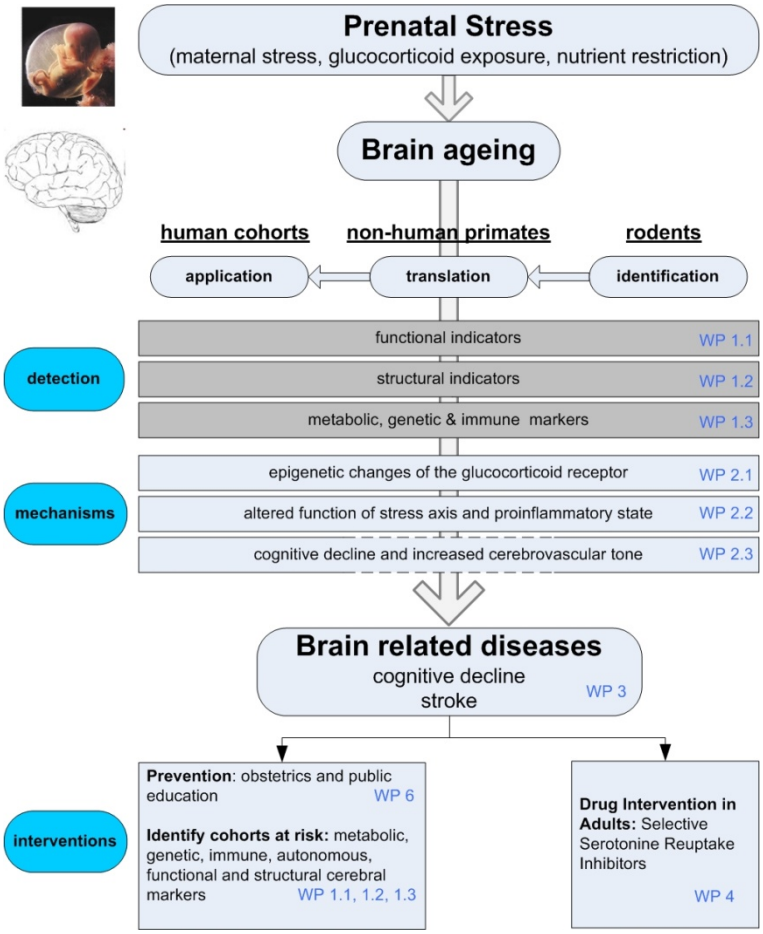

Fig. 3.: *Overall concept of the workplan*

(i) In order to link human development and ageing, we incorporated well defined existing cohorts since the establishment of such cohorts was not feasible within the time frame of the project.

(ii) In order to analyse mechanisms linking development and ageing, we needed appropriate experimental approaches, i.e. control and transgenic rodents. We, therefore, incorporated a strong translational approach in which we made sure that we determine comparable parameters in different species.

(iii) In order to develop predictors/markers for early brain aging we incorporated innovative methodological strategies to define brain ageing. We ensured that comparable testing and evaluation strategies are employed in the different human and experimental cohorts.

(iv) Whilst early intervention is desirable (prevention of stress in the womb), it is also desirable to identify treatment strategies for those subjects who have increased risk for early brain ageing and age-associated brain disease. Moreover, there are millions of people in the EU and all over the world, who are already struggling with the effects of prenatal stress on their health. This population also needs targeted interventions.

Based on these requirements, our project followed an interdisciplinary, highly standardized and translational approach. Our consortium was composed of participants with specific complementary expertise in the topics needed to carry out this interdisciplinary project.

We have selected some of the best characterized human cohorts at different ages from early childhood to the elderly that have been exposed to prenatal stress to examine and compare the effects of major prenatal stress factors (maternal stress, glucocorticoid exposure and nutrient restriction) on structural and functional brain development and ageing, and on the predisposition for brain-related diseases (early cognitive decline and stroke).

Rodents and transgenic mice have been used (i) to examine the underlying mechanisms of early brain ageing; (ii) to examine the age when the effects become phenotypically apparent; (iii) to examine the mechanisms of predisposition for brain-related diseases such as early cognitive decline and stroke; (iv) to establish an interventional approach.

Non-human primate cohorts were used to ensure translational power with the animal model closest to the human. We have selected a participant from the United States whose research group is the only one in the world owning adult non-human primate cohorts that were prospectively exposed to different prenatal stressors (glucocorticoid exposure and nutrient restriction) and were dedicated to study Developmental Programming of Health and Disease in later life.

We have chosen a strong gender-related approach because the response of the placenta to prenatal stress is sexually dimorphic and stress sensitivity during later life varies between sexes.

3. Description of the main S & T results/foregrounds

3.1. Overview

Please note: Not all results that not have been published need to be considered as preliminary results.

Cohorts

The *BrainAGE* consortium had unique access to well defined human, non-human primate and rodent cohorts across the lifespan that had been exposed to different types of prenatal stress.

Rodents

We bred rodents under standardized conditions and subjected these to two distinct types of prenatal stress: social stress and glucocorticoid treatment with dexamethasone at the dose used clinically in about 10% of all pregnancies to enhance fetal lung maturation in threatened premature labour, and maternal undernutrition. To clarify the function of glucocorticoid receptors on prenatal stress in stroke, we used mouse strains with conditional and functional selective mutations of the glucocorticoid receptors.

Non-human-primates

To translate our results from the rodents to humans and to validate the findings in our human studies, we have unique access to baboon cohorts of different ages that are based in San Antonio, Texas. The baboon is the most extensively studied non-human primate that is closest to human development and ageing. We have developed cohorts of baboons exposed to the same prenatal stressors as our human cohorts but in a standardized manner by means of moderate nutrient restriction and maternal glucocorticoids, i.e., 30% global nutrient restriction and maternal betamethasone treatment at the dose used clinically to enhance fetal lung maturation in threatened premature labour. It is important to note that up to 80% of individuals exposed to inappropriate levels of glucocorticoids as a result of maternal administration of glucocorticoids in threatened preterm labor do not deliver prematurely. The relevance of the nutrient restricted cohort is underscored by the fact that neither malnutrition in the Dutch famine cohort nor the 30% global nutrient restriction in the baboon induced a major reduction in birth weight. Furthermore, the effects on cognition were independent of size at birth. These cohorts provide an ideal translational setting between rodents and humans that is unique world-wide.

Humans

Prenatally stressed cohort, 9mo and 4y of age: Examination of this prospective longitudinal cohort began in 2009 in Tilburg (The Netherlands) in 170 mothers, aged between18 to 35y. The participants completed standardized stress and anxiety questionnaires at 10-14w, 15-22w and at 30-37w of pregnancy.

Prenatal glucocorticoid treated cohort, 14y of age: This prospective longitudinal cohort is based in Jena, Germany. Children in this cohort were born from mothers who had received either a single course (n=22) or multiple courses (n=17) of betamethasone (2x8mg 24h apart) to enhance fetal lung maturation between 26wk and 32wk gestation and delivered after 36wk. Babies had normal birth weight and did not need neonatal intensive care at the . Controls were matched for gender, age and gestational length.

Prenatally stressed cohort, 28-29y of age: This cohort is based in Leuven, Belgium. Offspring from 86 pregnant women s aged18 to 30y old have prospectively and longitudinally been followed up since 1986. The 86 pregnant women completed standardized stress and anxiety questionnaires at 12-22w, 23-31w and at 33-40w of pregnancy. The questionnaires differentiated between high and low stressed (anxious) mothers.

Dutch famine cohort, 68y of age: While famine is sadly not uncommon in many parts of the world, studying effects of undernutrition during specific periods of pregnancy is hampered by the fact that undernutrition is usually not restricted to pregnancy alone, and effects of chronic undernutrition and accompanying problems of infection complicate the situation. What is unusual about the Dutch famine is; firstly, the famine was imposed on a previously well-nourished population; secondly, there was a sudden onset and relief from the famine; and, thirdly, despite the adversities of the war, midwives and doctors continued to offer professional obstetric care and kept detailed records of the course of pregnancy, the delivery and the size and health of the baby at birth. Furthermore, detailed information is available on the weekly rations for the people in Amsterdam, and because birth records were kept of babies born in the Wilhelmina Gasthuis (one of the main teaching hospitals at the time) in Amsterdam, those born around the time of the famine could be traced and the long-term effects of famine could be studied. All of these characteristics make the Dutch famine a unique counterpart for animal models that examine the effects of restricted maternal nutrition during different stages of gestation.

A group of 2414 term singletons born between November 1943 and February 1947 in the Wilhelmina Gasthuis in Amsterdam for whom detailed birth records were available were traced. We defined famine exposure according to the daily official food rations for adults. A person was considered to be prenatally exposed to famine if the average daily ration for adults during any 13 week period of gestation was less than 1000 calories. We defined periods of 16 weeks each to differentiate between those who were exposed to famine in late gestation, mid gestation and in early gestation. All cohort members born before the famine or conceived after the famine served as the control group. The medical birth records provided information about the mother, the course of the pregnancy, and the size of both the baby and the placenta at birth. Socio-economic status of the mothers was based on the occupation of the head of the family at the time of delivery.

Main results

Following exposure to different prenatal stressors, we found increased stress sensitivity during later life and altered structural brain development and ageing resulting in early cognitive decline in both animal and human cohort studies. These effects are in part gender-related. Mechanisms for these effects include epigenetic mechanisms, developmental programming of increased stress sensitivity, and changes in lipid metabolism during later life. With regard to age-related brain diseases, prenatal stress worsens stroke outcome in both male and female rodents corresponding to the time of exposure to prenatal stress. The decreased stroke outcome is, at least partly, due to an increased cerebral vascular tone. A detailed study in rodents revealed that vulnerability to stress depends on the time of exposure during pregnancy. The vulnerable time periods for stress during pregnancy differed with regard to the occurrence of altered stress sensitivity and depressive behavior in later life.

The *BrainAGE* consortium has developed innovative methods to determine biological brain age in contrast to chronological age. These methods include the MRI-based structural *BrainAge* score and quantification of the age-related decline of cognitive functioning which is centred on a novel neuro-cognitive approach. These approaches allow the assessment of the information processing capacity of the cerebral attention network as a marker to quantify aberrations of brain development and ageing. Using this approach in humans, autonomic dysfunction as another marker of subtle aberrations in brain development is already detectable in children following prenatal maternal psychological stress and glucocorticoid exposure suggesting that autonomic dysfunction is a

sensitive marker of altered stress sensitivity during childhood. Other promising biomarkers that correlate with age are metabolic changes in lipid metabolism.

BrainAGE explored the effects of an early interventional approach based on lifestyle changes of the pregnant mother. For those subjects who have already an increased risk for age-associated brain diseases, we showed in rodents that pharmacological intervention with the serotonin re-uptake inhibitor citalopram during later life reduces impact of prenatal stress on stroke outcome. This effect is, at least in part, due to normalisation of increased vasotone.

One major outcome of *BrainAGE* is the special journal issue on the subject of "Prenatal Stress and Brain Disorders in Later Life" in the high impact journal *Neuroscience and Biobehavioral Reviews (IF 8.9)*. This special issue gives an overview of the current literature in this topic including the outcome of the present project.

Peer reviewed scientific publications

1. Franke K, Bublak P, Hoyer D, Billiet T, Gaser C, Witte OW, Schwab M. Noninvasive structural and functional markers of brain development and aging in humans. Neurosci Biobehav Rev. 2017 under review.
2. Franke K, van den Berg BRH, de Rooij SR, Roseboom TJ, Nathanielsz PW, Schwab M. Effects of Prenatal Stress on Structural Brain Development and Aging in Humans. Neurosci Biobehav Rev. 2017 under review.
3. Müller JJ, Antonow-Schlorke I, Kroegel N, Rupprecht S, Rakers F, Witte OW, Schwab M. Maternal psychological stress and offspring mental health – the role of cardiovascular and cerebrovascular programming. Neurosci Biobehav Rev. 2017 under revision.
4. van den Berg BRH, van den Heuvel MI, Lahti M, Braeken M, de Rooij SR, Entringer S, Hoyer D, Roseboom TJ, Räikkönen K, King S, Schwab M. Prenatal developmental origins of behavior and mental health: the influence of maternal stress in pregnancy. Neurosci Biobehav Rev. 2017 in press.
5. Vettorazzi S, TuckermannJ, Schwab M. Prenatal stress, glucocorticoid signaling and the brain. Neurosci Biobehav Rev. 2017 under review.

3.2. Link between prenatal stress and brain ageing

3.2.1. Effects of prenatal stress on structural brain development and ageing

Summary

Prenatal stress induces aberrations in structural brain development and early brain ageing which are in part gender-related.

Determination of biological structural brain age

Biological structural brain age in contrast to chronological age is an intuitive measure to quantify aberration in brain development and ageing. We have developed an innovative method to determine structural brain age using computational morphometry of anatomical T1-weighted MR images. This method is based on the normal aging pattern in the brain that has been determined in a large sample of healthy subjects. Using this normal aging pattern, a machine-learning algorithm is trained to predict the age of a given subject's brain. The difference between the predicted and true age results in a *BrainAGE* score. This method can be seen and interpreted as a measure of the number of years the brain structure of a subject differs from the expected normal ageing process. This brain age gap estimation framework allows the prediction of brain age with an error of between 1.3y (for children) and 5y (for adults) (Fig. 4). A major advantage of this approach allows age estimation at the level of a single subject. According to our current understanding, this is the first and only method that allows firstly the estimation of structural brain age per se and secondly it allows the estimation even across different MRI scanners. Importantly, the *BrainAGE* score is closely related to cognitive function (Loewe, PLoS One 2016).

During the life-time of the *BrainAGE*, we have adapted the brain age gap estimation framework to non-human primate and rat brain images. We used a deformation-based morphometry approach which we had previously developed for use with human brain data. Deformation-based morphometry is a useful technique to detect structural differences over the entire brain since it analyses positional differences between every voxel and a reference brain. The idea was to use nonlinear registration to deform one brain onto a reference brain. Following this registration, morphological differences between both brains are minimized and the deformations encode information relating to these differences. The Jacobian determinant can be finally used to calculate local volume changes at every voxel. Our deformation-based morphometry approach was validated in an earlier study by comparing the deformation fields and its Jacobian determinant with manually traced ventricular volumes.

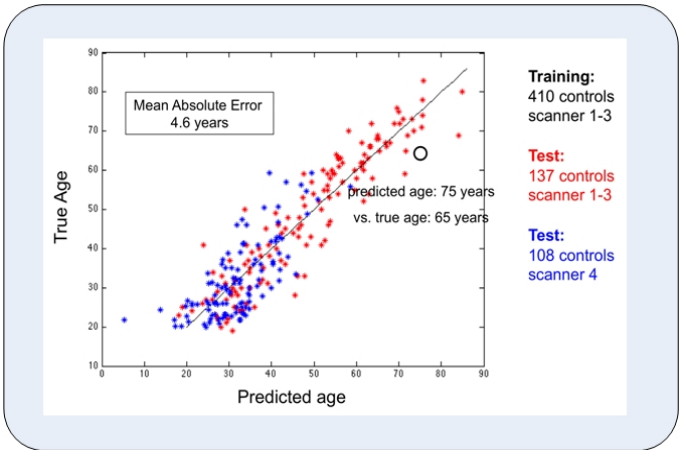

Fig. 4. *Estimation of structural brain age.*

Biological structural brain age (predicted age) and chronological age (true age) are shown for two different samples (red stars: same MR-scanners as used for training; blue stars: different MR-scanner not used for training). The black circle indicates an example for an Alzheimer's patient with a numerical age of 65y and predicted age of 75y.

This approach resulted in a very high correlation between the estimated brain age and chronological age in rats and non-human primates, e.g. in rats with r=0.97 (R^2=0.94) and a mean absolute error of 47 days in rats (Fig. 5).

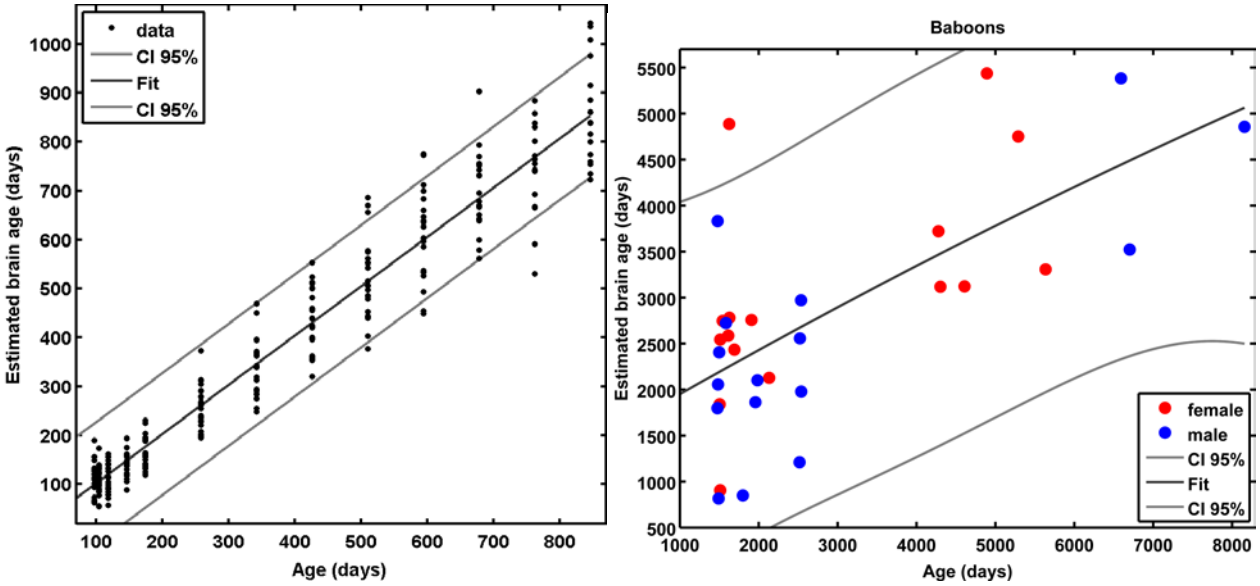

Fig. 5. *Adaptation of the BrainAGE framework to rats (left) and non-human primates (right).*

Note the high correlation between estimated brain age and chronological age.

We also used different MRI techniques to assess the heterogeneous white matter microstructural changes associated with aging in addition to brain atrophy. Since univariate MRI measures to specific microstructural features are confounded by a lack of specificity and, in the case of novel measures, a lack of studies characterizing their behavior in healthy tissue. Hence, using a multimodal MRI approach, we quantified whole-brain and regional age-related differences in both established (diffusion tensor imaging) and novel diffusion MRI metrics (diffusion kurtosis imaging, neurite orientation dispersion and density imaging) as well as in the myelin-specific MET2

technique in a prospective sample of healthy individuals. Diffusion tensor imaging and neurite orientation dispersion and density imaging appear to be most sensitive to life span effects during young to mid-adulthood (Billiet, Neurobiol Aging 2015). *BrainAGE* thus contributes valuable age–related normative data for future studies using these techniques and demonstrates the added value of using multiparametric MRI data for assessing age-related white matter microstructural changes that predate atrophy.

Effects of prenatal stress on structural brain development and ageing

Rats

We used rats to explore the effects of prenatal stress on structural brain development and ageing systematically by means of two stressors; treatment with glucocorticoids (dexamethasone) and nutrient restriction. Prenatal glucocorticoid exposure led to complex changes of the trajectory of brain development and ageing (paper in preparation).

Non-human primates

We used non-human primates, an animal model that is close to the human situation, to study the effects of prenatal stress on structural brain development and ageing under standardized conditions. Using the *BrainAge* score, we found that exposure to 30% maternal nutrient restriction during gestation resulted in premature structural brain aging by +2.7y in young adult female baboons (aged 4–7y) (Franke, Front Aging Neurosci 2017). Maternal treatment with betamethasone led to structural premature brain aging by +1.3y ($p<0.05$) in adult male baboons (10y; human equivalent 40y) (paper in preparation).

Humans

Prenatally glucocorticoid treated cohort, 14y of age: The offspring of mothers who were treated with betamethasone during the 3rd trimester also showed changes in the trajectory of structural brain development.

Prenatally stressed cohort, 28-29y of age: Young adults exposed to a high level of maternal anxiety between 12 and 22wk of pregnancy showed a less mature structural brain development than young adults exposed to a low or medium level of maternal anxiety during this period (paper in preparation). There is also a significant association between maternal anxiety between 12 and 22wk of pregnancy and the structural *BrainAge* score at the age of 28-29y (paper in preparation).

Dutch famine cohort, 68y of age: Males but not female subjects conceived during the Dutch famine and were thus exposed to nutrient restriction during their earliest prenatal development had a smaller intracranial and total brain volume compared to unexposed subjects. Thus, prenatal nutrient restriction permanently affects brain size (de Rooij SR, Brain 2016). Further, analyses showed increased *BrainAGE* scores compared to subjects who were conceived after the Dutch famine, i.e. they have a brain age that is 1.66 ± 4.62 years older than a normal collective (Franke, Neuroimaging 2017).

Peer reviewed scientific publications

6. Billiet T, Vandenbulcke M, Mädler B, Peeters R, Dhollander T, Zhang H, Deprez S, Van den Bergh BRH, Sunaert S, Emsell L. Age-related microstructural changes quantified using myelin water imaging and advanced diffusion MRI, Neurobiol Aging. 2015 36(6): 2107-21.
7. de Rooij SR, Caan MW, Swaab DF, Nederveen AJ, Majoie CB, Schwab M, Painter RC, Roseboom TJ. Prenatal famine exposure has sex-specific effects on brain size. Brain. 2016 Aug;139(Pt 8):2136-42.
8. de Rooij SR, Roseboom TJ. The developmental origins of ageing study protocol for the Dutch famine birth cohort study on ageing. BMJ Open. 2013 Jun 20;3(6). pii: e003167
9. Franke K, Clarke GD, Dahnke R, Gaser C, Kuo AH, Li C, Schwab M, Nathanielsz PW. Premature Brain Aging in Baboons Resulting from Moderate Fetal Undernutrition. Front Aging Neurosci. 2017 Apr 11;9:92.

10. Franke K, Gaser C, de Rooij SR, Schwab M, Roseboom TJR. In-vivo evidence for premature brain aging in old men prenatally exposed to maternal nutrient restriction. NeuroImage. 2017 under revision.

11. Franke K, Hagemann G, Schleussner E, Gaser C. Changes of individual BrainAGE during the course of the menstrual cycle. NeuroImage. 2015 115:1–6.

12. Franke K, Dahnke R, Clarke GD, Kuo A, Li C, Nathanielsz PW, Schwab M, Gaser C. MRI based biomarker for brain aging in rodents and non-human primates. 2016 International Workshop on Pattern Recognition in Neuroimaging (PRNI), Trento, 2016, pp. 1-4.

13. Loewe, L., Gaser, C., Franke, K., for the Alzheimer's Disease Neuroimaging Initiative (2016). The effect of the APOE genotype on individual BrainAGE in normal aging, mild cognitive impairment, and Alzheimer's disease. PlosOne 11(7): e0157514

3.2.2. Effects of prenatal stress on functional brain development and ageing

Summary

Prenatal stress induces aberrations in cognitive brain development, behavioural abnormalities and early cognitive decline which are in part gender-related.

Determination of the biological functional brain age

Based on a neural theory of visual attention (NTVA) that bridges behavioural and neurophysiological research, we developed an innovative neuro-cognitive approach to assess the information processing capacity of the cerebral attention network in humans. The method provides distinct parameters for quantifying speed of information processing, temporary information storage, and aspects of top-down related information selection. It, therefore grasps core aspects of attention, working memory, and executive function that are typically required for managing complex cognitive tasks such as planning, problem solving, and decision making. In particular using the NTVA-based approach, we can map the age-related decline of cognitive functioning and assess the cognitive state bordering between normal aging and dementia, termed 'mild cognitive impairment'. Outstanding in its theoretical foundation, the NTVA-based approach can assess unconfounded data in separate attentional components using the same task, and in this way avoids the problems of data interpretation that typically arise when using a battery of conventional tests that differ with respect to stimulus and response requirements.

Effects of prenatal stress on brain function in relation to age

Rats

We used rats to systematically explore the effects of prenatal stress on behaviour during brain development and ageing. After prenatal dexamethasone exposure and following postnatal stress, only male rats showed more depressive and less active behaviour at puberty (3mo of age) compared to controls. Interestingly, female rats started to develop depressive behaviour post-puberty, whereas the behaviour of male rats changed to less depressive and more active behaviour. These behavioural changes could not be detected any longer in both genders during old age (publication in preparation). In order to obtain a better understanding of the mechanisms underlying the programming effects of prenatal glucocorticoids on behaviour, we examined the effects of prenatal glucocorticoids on functional brain development using somatosensory evoked potential in the sheep fetus in utero. Antenatal glucocorticoids affect structural and functional development of the somatosensory system with specific effects at subcortical level (Anegroaie, Acta Physiol 2017).

Non-human primates

The non-human primate offspring tested were delivered by mothers fed ad libitum throughout gestation and lactation (controls) or received 70% of food eaten by controls. Our analyses indicate that offspring of nutrient restricted mothers at 3—5y and 8–11y of age (human equivalent 12-20y and 32–44y) show less motivation and impaired working memory in a gender-related fashion. They also showed behavioral abnormalities (Huber, J Med Primatol 2015; Huber, J Med Primatol 2015). A future course of action that is planned includes using these data as preliminary data to submit a proposal to the NIH to continue the follow up at 13–16y (human equivalent 42–64y) and 17–20y (68–80y). Thus, we are in the process of obtaining a full life course aging phenotype.

Humans

Prenatally stressed cohort, 9mo and 4y of age: Mindfulness decreases psychological distress during pregnancy (Braeken, Psychophysiology 2017). Offspring of more mindful mothers displayed less negative social-emotional behaviour compared to offspring of less mindful mothers at 9mo of age (Braeken, Biol Psychol. 2015; Braeken, Psychophysiology 2017). This was associated with less infant self-regulation problems and less infant negative affectivity (van den Heuvel, Early Human Dev 2015). Our event-related potential studies indicate that these infants also devote fewer attentional resources to frequently occurring irrelevant sounds (van den Heuvel, Soc Cogn Affect Neurosci 2015; van den Heuvel, Int J Psychophysiol 2015). A pregnant woman scoring high on mindfulness is a resilient person. Resilience can be viewed as a defense mechanism, which enables people to thrive in the face of adversity and improving resilience may be an important target for treatment and prophylaxis. On the other hand, prenatal exposure to maternal anxiety is related to more extensive processing of fear-related stimuli (Otte, Brain Cognition 2015). Infants generally dedicate more processing capacities to potentially threatening than to non-threatening stimuli (Otte, Brain Cognition 2015). Similarly, cognitive tests in 4y olds indicate that contextual emotional processing and cognitive control are altered in children of high anxiety mothers; they show signs of maladaptive fear (van den Heuvel, Dev Sci 2017).

Prenatally glucocorticoid treated cohort, 14y of age: Analysis of the cerebral information processing capacity in the cohort revealed that subjects showed reduced temporary information storage compared to controls suggesting aberration in cerebral information processing (publication in preparation).

Prenatally stressed cohort, 28-29y of age: Offspring of high anxiety mothers showed a decreased iconic memory suggesting aberration in cerebral information processing (publication in preparation). In fMRI studies, we found neurobiological correlates for the association between a deficit in endogenous cognitive control in adolescence and exposure to high maternal anxiety in the prenatal life period (Mennes, bioTvix 2016).

Dutch famine cohort, 68y of age: Prenatal exposure to famine was associated with cognitive decline: Individuals who had been exposed to famine during early gestation performed worse on a selective attention task. Interestingly, selective attention is a cognitive ability that is amongst the first to decline with age and has been shown to be a strong predictor for conversion to Alzheimer's' disease even before memory deficits appear. Prenatal undernutrition was also associated with decreased physical strength in men in later life, but not in women (Bleker, J Gerontol A Biol Sci Med Sci 2016). Our findings provide further evidence for the hypothesis that prenatal undernutrition may lead to an accelerated aging process in humans.

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3.3. Effects of prenatal stress on age-related brain diseases: cardiovascular and cerebrovascular diseases

Summary

We found an association between prenatal stress and stroke outcome in rodents but we couldn't show increased stroke prevalence or mortality in the Dutch famine cohort ue to the low numbers of stroke cases in the cohort.

Association of prenatal stress and stroke outcome in rodents

Prenatal stress worsens infarct size and stroke outcome in both male and female rodents in relation to the time of prenatal glucocorticoid exposure. The effect can already be detected during adulthood and is also seen during older age (publication in preparation). Methods to test the functional outcome have already been published (Antonow-Schlorke et al. 2013).

Prenatal stress and prevalence of stroke in humans

We did not find differences in stroke prevalence and stroke mortality after prenatal famine exposure in the Dutch famine cohort. This may be due to the low numbers of stroke cases in the cohort, which limited our statistical power to detect subtle differences. Based on our current analyses, we can exclude large effects of prenatal famine exposure on stroke prevalence. Future studies are needed to investigate potentially more subtle differences once the cohort ages and the numbers of stroke patients inevitably increases (Horenblas, J DOHaD 2017).

Effects of prenatal stress on cardiovascular function

We additionally studied aging of the heart to correlate with brain age in non-human primates since the MRI images obtained from the baboons incorporated thoracic images; Clearly, cardiovascular function and brain aging are linked to cerebrovascular diseases such as stroke. We prospectively show in the non-human primate model (6y; human equivalent 25y) that a 30% maternal nutrient restriction results in long-term programming of poor offspring cardiovascular health and a premature ageing phenotype in the heart (Kuo, J Physiol 2017; Kuo, J Physiol 2017). These results provide an extremely valuable base for designing and understanding human studies in the field of developing programming.

Peer reviewed scientific publications

33. Antonow-Schlorke I, Ehrhardt J, Knieling M. Modification of the ladder rung walking task-new options for analysis of skilled movements. Stroke Res Treat. 2013; 2013 :418627.

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3.3. Mechanisms of the link between prenatal stress and brain ageing

3.3.1. Epigenetic changes

Summary

DNA methylation patterns of different Nr3c1 promoters and imprinting control region (ICR) of the H19/IGF2 locus from rats show a complex pattern of locus-, tissue-, sex- and age-specific DNA methylation. Prenatal stress specifically modifies methylation of the rat Nr3c1 and Igf2/H19 ICR sex, tissue and age. However, we did not find striking differences in the DNA methylation in our human cohorts.

Determination of epigenetic changes of the glucocorticoid receptor gene (NR3C1) promoter

DNA methylation (mCpG) of the multiple promoters of the glucocorticoid receptor gene (*NR3C1*) was assessed by bisulfite next-generation sequencing (NGS) in a highly parallel approach. This method allows inspection of individual CpG sites from Illumina sequencing of PCR amplicons. To determine the *NR3C1* promoter methylation, bisulphite modification of DNA and bisulphite-specific nested PCR was established with complete coverage of the -5kb promoter region, one PCR product at the upstream -30kb promoter region, and one PCR product within the imprinting control region upstream of the H19 gene as positive control (Fig. 6). Fourteen PCR products were selected for the analysis using cloning and Sanger sequencing – the established gold-standard technique. Three PCR products were selected for development and analysis by means of pyrosequencing. To achieve high throughput, a hierarchical multiplexing approach was developed. We used innovative approaches for integrative statistical analysis (CuCompare) and global LINE methylation analysis (motif-based).

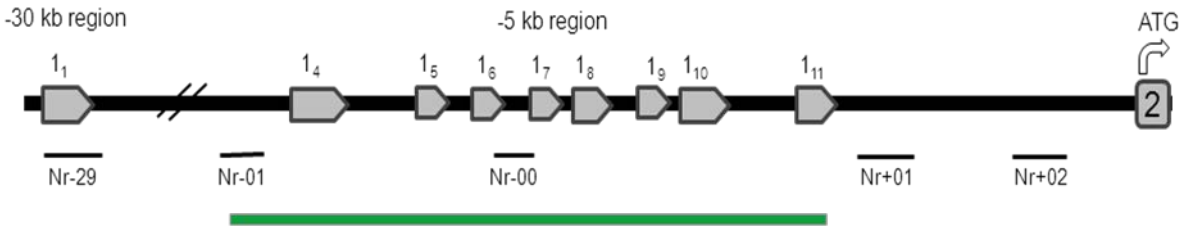

Fig. 6. *Promoter structure of rat Nr3c1 and analyzed amplicons/loci.*

The location of the alternative first exons (1_1, 1_4, 1_5, 1_6, 1_7, 1_8, 1_9, 1_{10}, 1_{11}), the second exon (containing the translation start site) and the analyzed amplicons (Nr-29/-01/-00/+01/+02) are shown. Green bar; CGI. Drawings are not to scale. Exon 1_1 and exons $1_{4\text{-}11}$, were annotated with GenBank accession BY121883 and AJ271870, respectively.

Global methylation analysis based on genome-wide repetitive elements

We performed global DNA methylation analyses in rats based on genome-wide repetitive elements (LINES). LINE retrotransposons with their hundreds of thousands of copies comprise about a fifth of mammalian genomes and harbor a significant part of methylated CpG sites of cellular methylomes. We analysed LINE methylation in rat tissues utilizing NGS of amplicons obtained from bisulfite-treated DNA. We further developed a novel analysis of the complex mixture of sequences derived from simultaneously amplifying multiple LINE loci using short sequence CpG-containing motifs.

Epigenetic modification of the glucocorticoid receptor gene (NR3C1) promoter and of the H19/IGF2 ICR

Prenatal glucocorticoid exposure did not introduce obvious alterations in the rat *NR3C1* promoter methylation (Fig. 7) but altered the H19/IGF2 ICR which controls expression of IGF2, the major

growth hormone during fetal development (Fig. 8). The finding that prenatal stress exerts its programming effects on cognition and behavior by changes inDNA methylation in the *H19/IGF2* locus rather than in the *NR3C1* promoter challenges current theories and will introduce a new level of understanding of the basic epigenetic mechanisms that contribute to the programming of cognitive and behavioural disorders. Our results also show that *NR3C1* promoter methylation is highly tissue specific in the rat (Fig. 7).

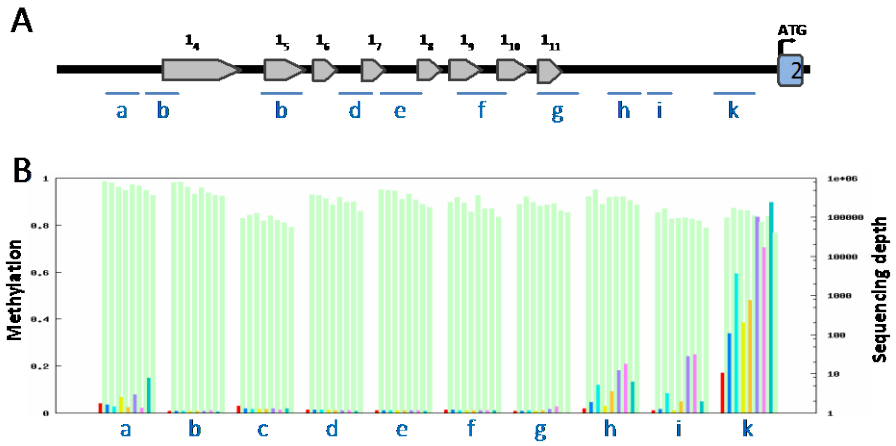

Fig. 7. *Tissue specific DNA methylation pattern of rat NR3C1.*

(**A**) 5 kb promoter region with eight alternative first exons and 10 analyzed amplicons a-k. (**B**) Methylation patterns of eight tissues determined by next-generation sequencing. Tissues in the order of appearance (from red to dark green columns): liver, cortex, hypothalamus, skin, hippocampus, adrenal, pituitary, blood; X-axis: amplicons a-k, left Y-axis: average methylation of all CpGs per amplicon. Right Y-axis: light green right to the respective methylation column; number of reads per amplicon.

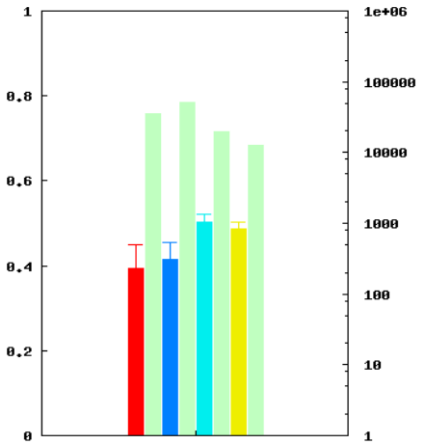

Fig. 8. *Differential methylation in the Igf2/H19 locus of a representative CpG in rat hypothalamus after prenatal GC treatment at different gestational ages determined by NGS.*

Left Y-axis: red - female control, blue - male control, cyan - male GC treated at E17/18, yellow - male GC treated at E19/20; n=3-4 animals, mean+SD. Right Y-axis: light green right to the respective methylation column; mean.

Epigenetic modification of the imprinting control region (ICR) of the H19/IGF2 locus

We found an unexpected result that prenatal stress changes the methylation of the imprinting control region (ICR) of the H19/IGF2 locus which controls expression of IGF2 and the H19 long non-protein-coding (lnc) RNA. IGF2 is the most important growth hormone during development and is strongly gender-specific in its expression. The ICR is paternally methylated and determines that H19 lncRNA is only expressed from the maternally-inherited chromosome and IGF2 from the paternally-inherited chromosome. Analysis of the imprinting center of Igf2/H19 complements the Nr3c1 promoter analysis and may explain (1) why early stress has the most pronounced effects on brain development and ageing in the period when glucocorticoid receptors are not yet expressed, and (2) the pronounced effects of moderate maternal nutrient restriction.

Tissue-, sex- and age-dependent DNA methylation of the Nr3c1 promoter and Igf2/H19 ICR

We analysed DNA methylation patterns of different Nr3c1 promoters and Igf2/H19 ICR in seven tissues of rats at 3, 9 and 24 months of age. We found a complex pattern of locus-, tissue-, sex- and age-specific DNA methylation. Tissue-specific methylation was most prominent at the shores of the Nr3c1 CpG island (CGI). Sex-specific differences in methylation peaked at 9 months. During aging, Nr3c1 predominantly displayed hypomethylation mainly in females and at shores, whereas hypermethylation occurred within the CGI. Igf2/H19 ICR exhibited age-related hypomethylation occurring mainly in males. Methylation patterns of Nr3c1 in the skin correlated with those in the cortex, hippocampus and hypothalamus. Skin may serve as proxy for methylation changes in central parts of the hypothalamo-pituitary-adrenal axis and hence for vulnerability to stress- and age-associated diseases. Thus, we providefor the first time in-depth insight into the complex DNA methylation changes of rat Nr3c1 and Igf2/H19 during ageing that are tissue- and sex-specific (Agba, Physiol Genomics 2017, Sahm, Mol Biol Evolution 2017).

Tissue-, sex- and age-dependent methylation of LINE L1 transposons

Rat tissues differ with regard to the extent of LINE methylation and exhibit sex- and age-dependent methylation patterns. Changes in LINE sequence distribution are age-dependent, and indicative of increased activity of retrotransposons during old age and the accumulation of somatic mutations at CpG sites during aging. This demonstrates that this novel approach is capable of providing - in high-throughput - quantitative insights into multiple aspects of the aging of DNA methylomes (Huse, Nucl Acid Res 2017).

Locus-, tissue- and sex-specific methylation changes after prenatal dexamethasone treatment

Rats

To study methylation changes at Nr3c1 and Igf2/H19 ICR due to prenatal stress exposure, pregnant rats were treated with dexamethasone at days 17 and 18 of gestation (E17/18). Changes were assessed in their 9mo old offspring by comparing with their age-match controls. Six amplicons and tissues involved in regulation of the hypothalamo-pituitary-adrenal axis (hippocampus, hypothalamus, pituitary gland, adrenal cortex) as well as a peripheral tissue (skin) were analysed. Figure 9 provides an overview of the observed methylation patterns in controls and E17/18 rats, and indicates the interplay of amplicon-, tissue- and sex-specific effects with prenatal dexamethasone treatment. Most pronounced changes in methylation levels were observed at the Igf2/H19 ICR in both male and female rats. We found that prenatal stress modifies sex-specific methylation in 9mo old rats (Fig. 10) and changes the methylation levels according to gender specificity (Fig. 111). Publication is in preparation.

Humans

Prenatally stressed cohort, 4y of age: The DNA source for the children was obtained as buccal swabs. The shores of the *NR3C1* associated CpG island, *IGF2/H19* ICR and LINE L1 elements were inspected for methylation. Distinct CpGs in L1 and *NR3c1* were found to have a higher level of methylation in boys. A significant association between maternal anxiety and the methylation of a distinct CpG at the upstream CGI shore was seen. For boys, there was a strong association between maternal anxiety and methylation of specific sites in the downstream CGI shore (van den Heuvel 2017).

Prenatally glucocorticoid treated cohort, 14y of age and prenatally stressed cohort, 28-29y of age: In-depth analyses of methylation of the shores of the NR3C1 associated CpG island, IGF2/H19 ICR and LINE L1 elements are ongoing. Publication of these results will follow.

Dutch famine cohort (AMC), 68y of age: We did not find differences in DNA methylation of the NR3C1 after famine exposure but differences in stress response were related to glucocorticoid receptor methylation (de Rooij, Psychoneuroendocrinology 2012).

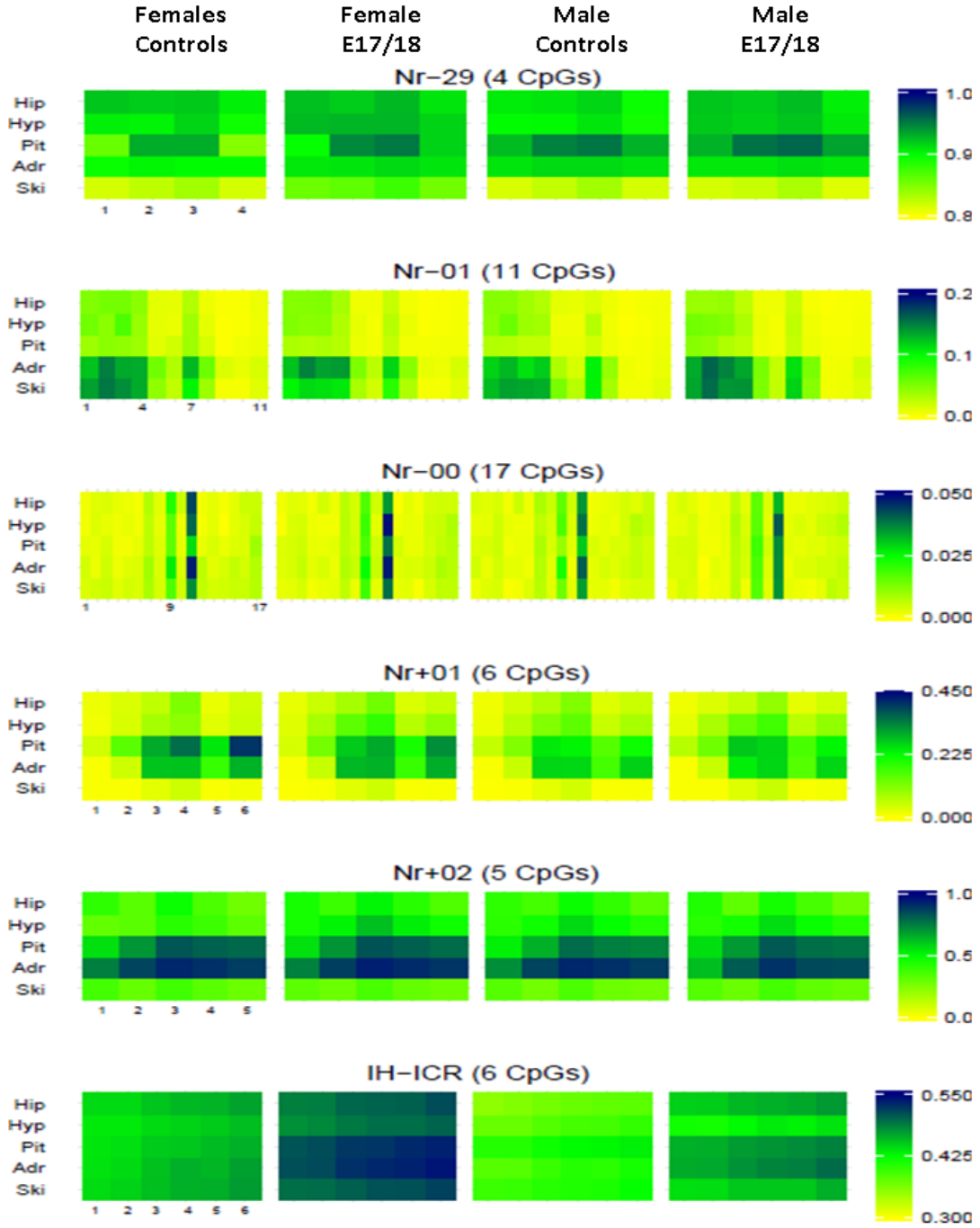

Fig 9. *Overview of methylation patterns of the analyzed loci and tissues in 9mo old controls and rats prenatally treated with glucocorticoids (dexamethasone) at E17/18.*

Top-down: amplicons; left half (females) and right half (males) showing the two groups (control and E17/18) as main columns. Sub columns: CpGs; rows: tissues (Hip - hippocampus, Hyp - hypothalamus, Pit - pituitary gland, Adr - adrenal cortex, Ski - skin); cell color: methylation rate of single CpG (mean of eight rats). Note that color scales are amplicon-specific, and sub columns represent CpGs per amplicon. Numbering of CpGs is shown on the left main column.

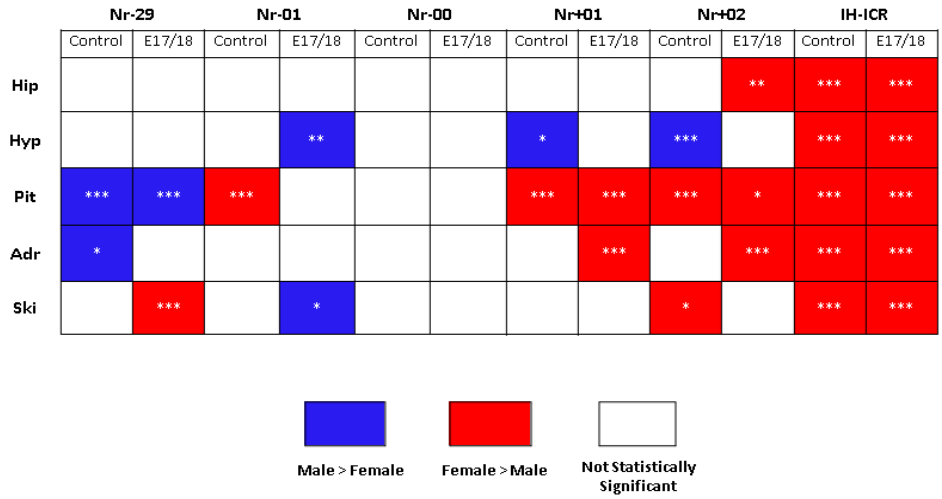

Fig. 10. *Sex-specific methylation in 9mo old controls and rats prenatally treated with glucocorticoids (dexamethasone) at E17/18.*

Columns - amplicons group-wise, rows - tissues (Hip - hippocampus, Hyp - hypothalamus, Pit pituitary, Adr - adrenal cortex, Ski - skin). Every block of two columns and five lines represents one amplicon (named at top). Stars and colored cells: statistically significant sex-differences, white cells: no statistically significant sex-differences. Directionality of sex methylation difference: blue – male > female, red – female > male. Significance levels: * <0.05, ** <0.01, *** <0.001, by CuCompare analysis and Mann-Whitney U test.

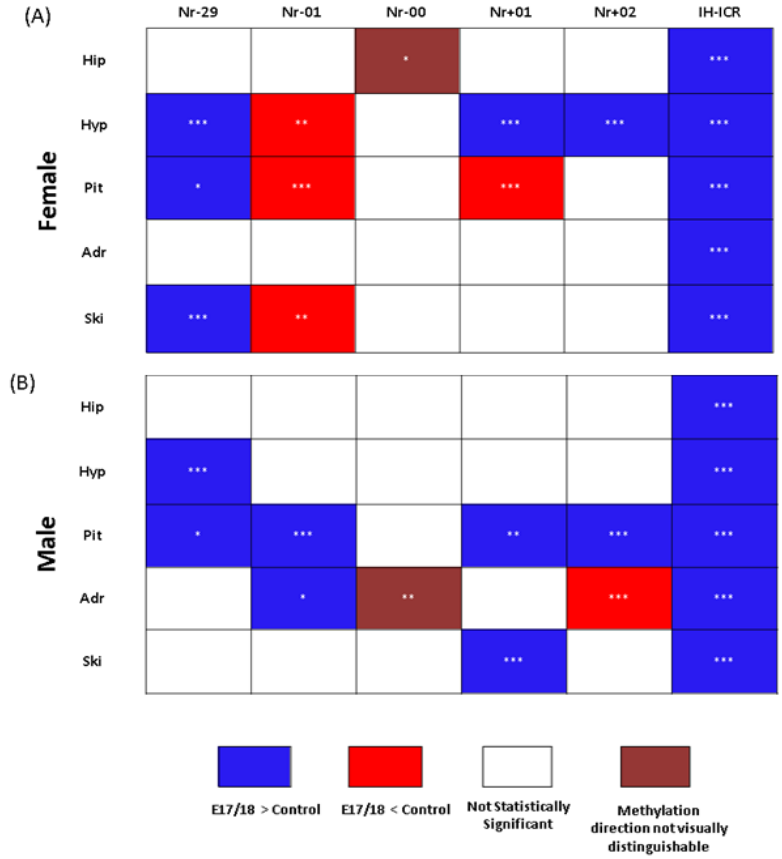

Fig. 11. *Methylation differences in 9mo old controls and rats prenatally treated with glucocorticoids (dexamethasone) at E17/18.*

Comparison to controls in females (A) and males (B). Columns – amplicons, rows – tissues (Hip - hippocampus, Hyp - hypothalamus, Pit - pituitary gland, Adr - adrenal cortex, Ski - skin). Every column and five lines represent one amplicon

(named at top). Stars and colored cells: statistically significant methylation differences, white cells: no statistically significant methylation differences. Directionality of methylation difference: blue - E17/18 > control; red – E17/18 < control; brown – indistinguishable direction of group difference. Significance levels: *<0.05, **<0.01, ***<0.001; FDR by CuCompare analysis.

Peer reviewed scientific publications

37. Agba OB, Lausser L, Huse K, Bergmeier C, Jahn N, Groth M, Bens M, Gall M, Witte OW, Kestler H, Schwab M, Platzer M. Tissue-, sex- and age-specific DNA methylation of rat glucocorticoid receptor gene promoter and insulin like growth factor 2 imprinting control region. Physiol Genomics 2017 under review.

38. de Rooij SR, Costello PM, Veenendaal MV, Lillycrop KA, Gluckman PD, Hanson MA, Painter RC, Roseboom TJ. Associations between DNA methylation of a glucocorticoid receptor promoter and acute stress responses in a large healthy adult population are largely explained by lifestyle and educational differences. Psychoneuroendocrinology. 2012 Jun;37(6):782-8.

39. Huse K, Lausser L, Sass M, Agba OB, Bergmeier C, Jahn N, Groth M, Koch P, Dziegelewska M, Reichelt M, Gall M, Kramer M, Witte OW, Schwab M, Kestler H, Platzer M. Analysis of global LINE DNA methylation by next generation sequencing reveals tissue- and gender-specificity and age-dependent changes in rats. Nucl Acid Res. 2017 under revision.

40. Sahm A, Bens M, Szafranski K, Holtze S, Groth M, Görlach M, Calkhoven C, Müller C, Kaether C, Schwab M, Cellerino A, Hildebrandt T, Burda H, Dammann P, Platzer M. Long-lived rodents reveal signatures of positive selection in genes associated with lifespan and eusociality. Mol Biol Evolution 2017 under review.

41. van den Heuvel MI et al. Epigenetic Modifications Induced by Maternal Anxiety During Pregnancy: DNA-Methylation in Buccal Cells of Preschoolers. Under submission.

3.3.2. Changes in stress sensitivity

Summary

Following exposure to the different prenatal stressors, we found increased stress sensitivity in animal and human cohort studies. A detailed study in the rodents showed that the vulnerability to stress depends on the time of exposure during pregnancy. The time periods for stress during pregnancy differ regarding the vulnerability for programming of stress sensitivity and depressive behavior. In humans, autonomic dysfunction is already detectable during childhood following prenatal maternal psychological stress and glucocorticoid exposure suggesting that autonomic dysfunction is a sensitive marker of altered stress sensitivity.

Determination of stress sensitivity

Stress sensitivity was determined by means of measuring the activity of the hypothalamo-pituitary-adrenal axis by means of quantifying cortisol levels. Further, we assessed the function of the autonomic nervous system using heart rate variability analysis and/or estimation of alpha-amylase in saliva during stress tests. We also estimated the activity of the 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2 in the brain. 11β-HSD2 is a high-affinity dehydrogenase that inactivates cortisol to cortisone. 11ß-HSD1 catalyses the reverse reaction regenerating active steroids, thus exposing brain cells to neurotoxic cortisol effects.

Effects of prenatal stress on stress sensitivity in later life

Rats

Stress sensitive periods during pregnancy

We identified vulnerable prenatal periods for programming of stress sensitivity in rodents by using prenatal dexamethasone treatment. Prenatal exposure to dexamethasone has more pronounced effects in terms of inducing hyper-responsiveness of the stress axis during later life in females than in males. In contrast, postnatal dexamethasone treatment or social stress exposure induces a hypo-responsive stress axis In both males and females.

The time periods for stress during pregnancy differ regarding the vulnerability for programming of stress sensitivity and depressive behavior although it is thought that depressive behavior is mediated by increased stress sensitivity. This means that mechanisms other than increased stress sensitivity such as a different trajectory of brain development mediate development of depressive behavior in later life. This view is supported by our findings on *H19/IGF2* gene locus methylation.

Age when altered stress sensitivity becomes detectable in later life

Following prenatal dexamethasone exposure, the stress axis is hypo-responsive during pre-puberty (3mo of age). During young adulthood, the stress axis becomes hyper-responsive in females but is still hypo-responsive in males. During old age, the stress axis also becomes hyper-responsive in males (publication in preparation).

Adversity of different prenatal stressors

The programming effects of prenatal GC exposure on the offspring stress axis depend not only on the time of exposure but also on the stressor. GC exposure at E17/18 is more powerful in programming a hyper-responsive stress axis than GC exposure at E15/16 or E19/20. In contrast, postnatal GC exposure at P4/5 induces a hypo-responsive stress axis whereas social stress has no programming effects. Conducting such explorations in a single laboratory is important since it ensures comparability of results, lending more validity to the results.

Non-human primates

We set-up a longitudinal life course profile of non-human primates for circulating cortisol levels from 6–20y of age (human equivalent 24–80y of age). Cortisol falls in a linear fashion over the life course (Yang, Aging 2017). This is the first study showing plasma cortisol levels right across the life course in any species. To determine what part of the neuroendocrine system in the brain causes this fall in cortisol, we conducted immunohistochemistry on the paraventricular nuclei of baboons (21–52y human equivalent). The data show an age related fall in arginine vasopressin with no fall in corticotropin-releasing hormone suggesting that in the aging process, AVP is a more important driver of the pituitary-adrenal axis than CRH. Of additional interest, the cortisol negative feedback system in the brain seems to increase with age. Glucocorticoid receptors are increased in the paraventricular nucleus which would result in increased negative feedback. Importantly, we have also shown a previously unknown increase in local production of cortisol within the hypothalamus which would also increase negative feedback (publication in preparation).

Data of non-human primates 6–20y of age (human equivalent 24–80y of age) show that the activity of 11βHSD1 increases with age in the brain suggesting that 11βHSD1 contributes to early cognitive decline by exposing neurons to increased cortisol levels. In addition, we show increased receptors (both glucocorticoid receptors (GR) and mineralocorticoid receptors (MR)) that will increase and potentially offset any decrease in circulating cortisol. This is therefore a complex system and our data have set the scene and show the key areas that require further investigation (publication in preparation).

Humans

Prenatally stressed cohort, 9mo and 4y of age: We could show autonomic dysfunction at 9mo of age after prenatal maternal stress (Braeken Psychophysiology 2016). In the 4y old, maternal anxiety in pregnancy compromises stress sensitivity of the offspring which may explain their risk for mental health problems (publication in preparation).

Prenatally glucocorticoid treated cohort, 14y of age: Similarly, subjects in this cohort showed increased sympathetic activity measured by alpha-amylase in saliva and increased overall autonomic activity by means of HRV analysis (publication in preparation). Glucocorticoid receptor resistance did not show any changes. Nor were gender differences in glucocorticoid receptor resistance detectable. There was also no correlation between glucocorticoid receptor resistance and cognitive function, serum cortisol concentration, or alpha-amylase in saliva as a measure of sympathetic activity (publication in preparation).

Prenatally stressed cohort, 28-29y of age: Subjects prenatally exposed to high maternal anxiety showed greater stress sensitivity than those exposed to low-to-medium levels (publication in preparation). A striking result is that the fetus seems to be most vulnerable between 12 and 22w of pregnancy; i.e., almost no significant effects were found for exposure during the other pregnancy periods. In addition, prenatal exposure to high maternal anxiety was associated with an increase in symptoms of breathlessness as a measure of autonomic dysfunction (von Leupoldt, Eur Respiratory J, in press).

Dutch famine cohort, 68y of age: Whilst nutrient restriction during early gestation induced increased activity of the hypothalamo-pituitary-adrenal axis at older age, prenatal undernutrition was not associated with autonomic dysfunction (de Rooij, Psychosom Med 2016).

Peer reviewed scientific publications

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43. von Leupoldt, A, Mangelschots, E, Niederstrasser, N G, Braeken, M, Billiet, T, Van den Bergh, BRH. Prenatal stress exposure is associated with increased dyspnea perception. Eur Respiratory J. 2017 in press.

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3.3.3 Metabolic changes

Summary

Prenatally stressed offspring show gender-dependent changes in lipid metabolism which may partly explain abnormalities in cognitive function and behaviour. Developmental programing via prenatal glucocorticoid exposure produces a mid-life metabolically obese but normal weight phenotype.

Determination of metabolic changes

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, especially when one considers, as we propose, that biological age is not only determined by genetic factors or lifestyle, but also by epigenetic determinants during early development, metabolic profiling can give an instantaneous snapshot of the physiology of that cell. Targeted metabolomics, i.e. the targeted analysis of a vast range of preselected metabolites, allows the rapid identification of metabolites and holds at the same time the potential to discover new metabolic dependencies and biochemical patterns not thought of before by a “classical” biochemical approach. This innovative strategy led to new insights into the effects of prenatal stress and age-related metabolic processes.

Rats

Rats showed gender-specific metabolite levels, most prominent for oxysterol metabolites, resulting in a clear separation into males and females (Fig. 12). Especially in female rats, nutrition of the mother showed effects on amino acid or biogenic amine composition in the offspring. In both genders, maternal nutrition affected carnitine levels indicating changes in fatty acid breakdown. Alterations in the overall lipid or bile acid metabolism were based on the age of the rats rather than on maternal nutrition status (paper in preparation).

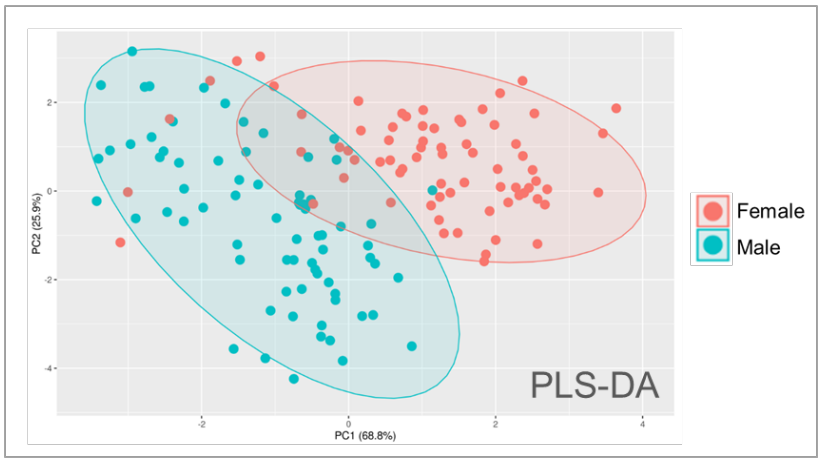

Fig. 12. *Based on sterol and oxysterol metabolites, Partial Least Squares Discrimination Analysis (PLS-DA) shows a gender-specific separation in rats.*

Non-human primates

We have conducted metabolomics of aging baboons between 13 and 19y of age (human equivalent 52–76y). These are the first findings for a comprehensive set of amino acids and lipid metabolites with age (publication in preparation). It is of interest that some metabolites change similarly in males and females and some differently. Male plasma sarcosine falls with age. This reduction in sarcosine may have a major effect on the increase in depressive behaviour with age that we have shown and report in our cognitive studies. The reduction in sarcosine tends to increase insulin resistance and decrease fatty acid metabolism predisposing to obesity. Lipid levels rise with age in plasma of males and females – potentially due to aging liver metabolism. These lipid changes may well impair brain function. Lipid levels in the brain rise with age in females but fall in males. The lipid increase in the brain in females parallels the changes in plasma lipids. Essential amino acids in the brain of males and females increase with age – potentially due to slowing of metabolism and turnover (paper in preparation).

In order to study the consequences of these metabolic changes we have structural consequences, we examined 10y old male offspring (human equivalent 40y) whose mothers were treated with the synthetic glucocorticoid betamethasone at doses and stages of fetal life equivalent to human obstetric practice to decrease premature labour. We quantified pericardial fat and hepatic lipid content with magnetic resonance imaging and spectroscopy. Pericardial fat thickness and hepatic fatty acids were increased following prenatal betamethasone treatment without birth weight or current body morphometric differences. Our results indicate that antenatal betamethasone therapy causes abnormal fat deposition and adult body composition in mid-life primate offspring (Kuo, Int J Obes (Lond) 2017). The concern raised is that this degree of pericardial and hepatic lipid accumulation can lead to harmful local lipotoxicity.

Humans

Prenatal glucocorticoid treated cohort, 14y of age: Prenatal stress was associated with a consistent decrease of blood lipids such as phosphatidylcholines (Fig. 13). Furthermore, several acylcarnitines were shown to be decreased in the prenatal stress group. In contrast, lysophosphatidylcholines, metabolites which are linked to inflammation and a variety of diseases, were elevated in the stressed group. These results clearly indicate an overall complex level of

change in lipid metabolism. Furthermore, different amino acids such as branched-chain amino acids as well as aromatic amino acids were elevated in the stress group (paper in preparation).

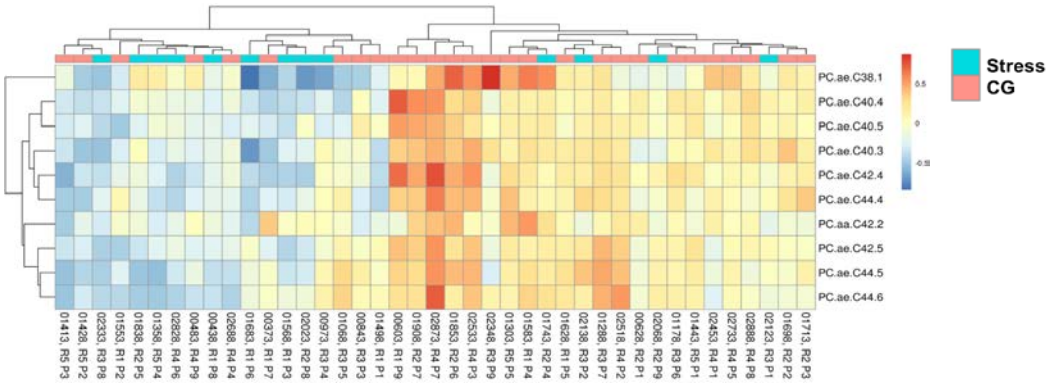

Fig. 13. *Hierarchical clustering analysis showing phosphatidylcholine-based separation of analysed cohorts (Stress - Prenatally glucocorticoid-treated cohort; CG – Control group).*

Prenatally stressed cohort, 28-29y of age: Multivariate statistical analysis by Partial Least Squares Discrimination Analysis revealed separation between high anxiety and low/medium anxiety groups in males and females (Fig. 14). Most prominently, male individuals were characterized by significantly higher levels of phosphatidylcholines, lysophosphatidylcholines and cortisone in the high anxiety group (Tab. 1). Increased acylcarnitines and sphingomyelins were found in the female high anxiety group. Interestingly, affected metabolites were substantially different in male and female individuals (paper in preparation).

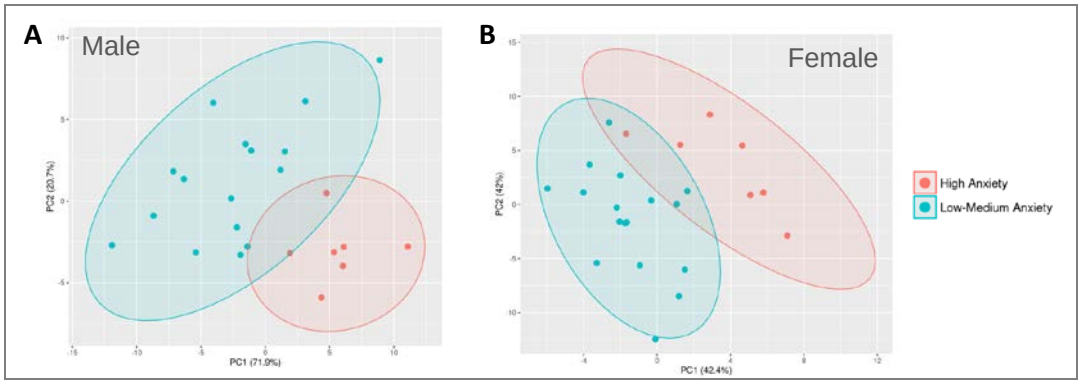

Fig. 14. *Partial Least Squares-Discrimination Analysis was applied on males (**A**) and females (**B**).* Shown are scoring plots and 95 % confidence interval ellipses for the two different groups, offspring derived either from high or low-medium anxiety mothers.

Metabolite Class	No. of Metabolites	
	Males	Females
Amino Acids & Biogenic Amines	3	2
Acylcarnitines	0	3
Glycerophospholipids	31	0
Sphingolipids	0	1
Steroids	1	1

Tab. 1. *Number of metabolites per metabolite class that are significantly different in the offspring depending on the maternal anxiety state during pregnancy (p<0.05).*

Dutch famine cohort, 68y of age: The metabolome was gender-specific (Fig. 15). Gender-specific data analysis, however, reduced the sample number per cohort, thus weakening the statistical power of the analysis. Upon gender-specific data evaluation, only few metabolic differences could

be identified with respect to stress exposure (Fig. 16). This may be explained, in part, by the age of the participating subjects in this long-running study. Only the healthiest subjects are still available while health-impaired and, thus, potentially stress-affected subjects may have dropped out. This results in a relatively homogenous study population, as reflected in the metabolite panel (publication in preparation).

Peer reviewed scientific publications

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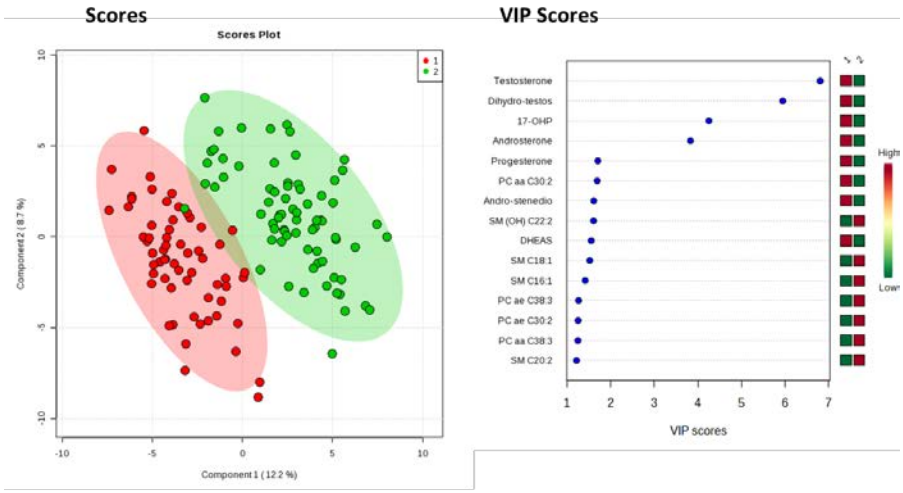
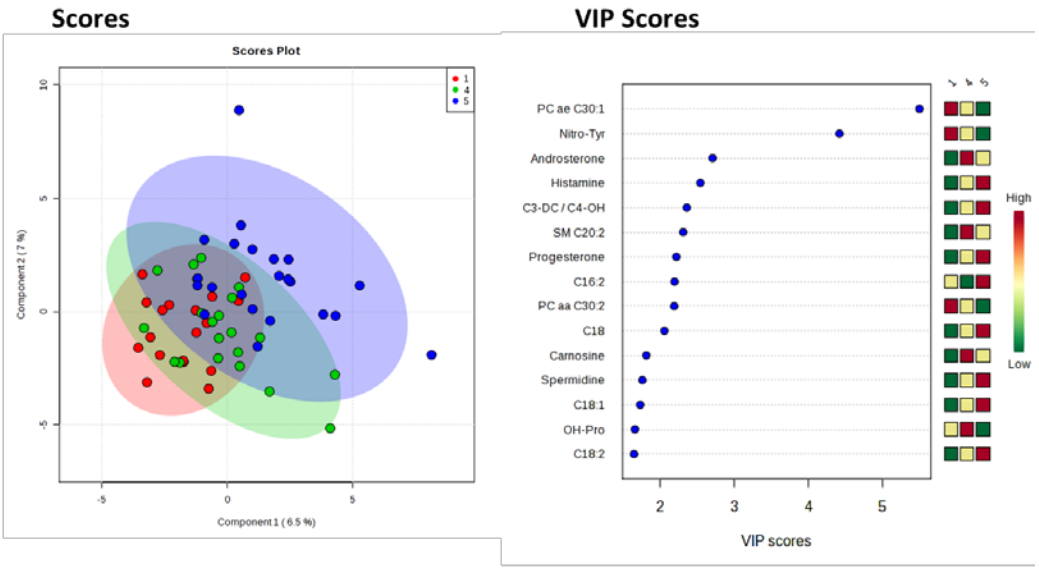


Metabolite	Fold Change	log2(FC)
Testosterone	21.872	4.451
Dihydro-testosterone	5.9582	2.5749
PC aa C30:2	5.2426	2.3903
Androsterone	4.1905	2.0671
17-OHP	3.5402	1.8238
SM C22:3	3.1762	1.6673

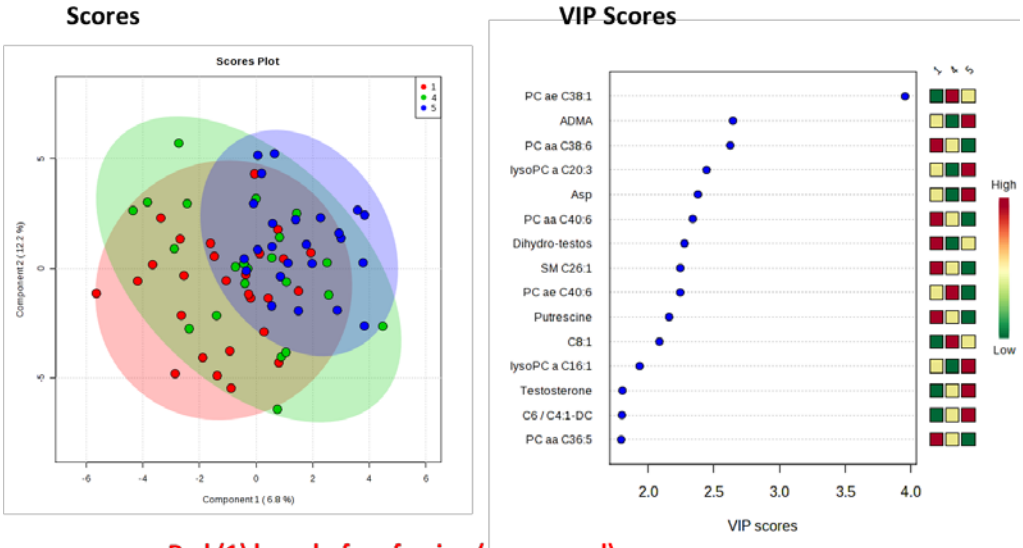
Fig. 15. *Sex-differences in metabolite content of the members of Dutch famine cohort shown by Partial Least Squares-Discrimination Analysis and fold change analysis.*

Male subjects: PLS-DA



Red (1) born before famine (unexposed)
Green (4) exposed in early gestation
Blue (5) conceived after famine (unexposed)

Female subjects: PLS-DA



Red (1) born before famine (unexposed)
Green (4) exposed in early gestation
Blue (5) conceived after famine (unexposed)

Fig. 16. *Effects of stress exposure in the Dutch famine cohort shown by Partial Least Squares-Discrimination Analysis (PLS-DA).*

3.3.4. Immune status

Summary

Cytokine plasma levels are not consistently related to prenatal stress.

Determination of cytokines and effects of prenatal stress

All commercially available test kits proved to be insensitive and/or unreliable for baseline cytokine measurements in healthy subjects. We found that baseline cytokine values were below the lower limit of detection. Therefore, we measured cytokine release from stimulated lymphocytes using the Luminex method. This method provides 1000-fold higher cytokine levels. Using this method, pro- and anti-inflammatory cytokines in the serum of 14y old offspring of glucocorticoid (betamethasone) treated mothers did not show any differences. Also gender differences were undetectable. The effect size of prenatal stress on the stimulated pro- and anti-inflammatory cytokine release was even smaller than the biological effect of sampling at different days and the methodical variance of the whole assay. Thus, we searched for possible explanations of the low effects of prenatal stress on the immune status. We attempted to prove the reliability of the Luminex measurement method by comparing it to the CE certified Cobas measurement method which has the highest proven reliability on the market although it only measures IL-6. The Cobas system showed consistently lower results than the Luminex system. In addition, the results of the Luminex method did not correlate with the Cobas method.

Intensive discussions with the manufacturers and scientists did not give any conclusion for these inconsistencies. It appeared that manufacturers and scientists in these companies did not subject the kits to the rigorous level of testing regarding reliability that we undertook. The unreliable measurements may explain the inconsistencies and differences of results on cytokine measurements from different labs.

3.4. Mechanisms of the link between prenatal stress and age-related brain diseases

3.4.1. Cerebrovascular reactivity

Summary

The decreased stroke outcome following prenatal stress is, at least partly, due to an increased cerebral vascular tone.

Prenatal stress and cerebrovascular reactivity during later life

Our hypothesis was that the negative stroke outcome in prenatally stressed rodents is determined due to an increased vascular tone. This possibly leads to a decreased perfusion of the penumbra of a stroke since the vessels cannot dilate maximally. We tested the cerebrovascular reactivity using several endothelium-dependent and independent vasoconstrictors and dilatators and found age-dependent changes in vasoreactivity in the cerebral circulation. Prenatal dexamethasone exposure increases vasoconstrictory and decreases vasodilatory responses in the renal and cerebral circulation (publication in preparation). In order to obtain a better understanding of the changes in vascular reactivity we examined the normal development of vascular function in the fetus (Müller, Am J Physiol 2017). Prenatal stress altered the trajectory of vascular development (paper in preparation).

Peer reviewed scientific publications

47. Müller J, Schwab M., Rosenrfeld CR, Antonow-Schlorke Nathanielsz PW, Rakers F, Schubert H, Witte OW, Rupprecht S. Fetal sheep mesenteric resistance arteries: functional and structural maturation. Am J Physiol. 2017 In press.

3.4.2. Changes of the glucocorticoid receptor-mediated control of cerebral inflammation

Summary

Changes in glucocorticoid receptor-mediated control of cerebral inflammation worsens stroke outcome.

Cell type-specific actions of glucocorticoid receptors involved in enhanced cerebral inflammation

We hypothesized that an insufficient glucocorticoid receptor-mediated suppression of secondary cerebral inflammation may increase cerebral infarction following stroke. Besides ischemic neuronal death, a secondary cerebral inflammation reaction following stroke is another major contributor to neuronal fade. Cortisol and the sympathetic-mediated release of norepinephrine inhibit Th1-mediated pro-inflammatory cytokine production giving way to the predominance of Th2-mediated anti-inflammatory cytokine production. This prevents an overwhelming cerebral inflammation. However, resistant glucocorticoid receptors as a result of a life-long hyperactive hypothalamo-pituitary-adrenal axis may insufficiently mediate the cortisol-induced inhibition of pro-inflammatory cytokine production. Moreover, glucocorticoids act via different pathways that are possibly changed by prenatal stress (Hartmann, Physiol Rev 2016; Hübner, Biol Chem 2015; Lim, Genome Res 2015; Liu, Sci Rep 2016; Mueller, Diabetes 2017; Vettorazzi, Nat Commun 2015). To explore the involvement of glucocorticoid receptor pathology in the decreased stroke outcome following prenatal stress, we used three genetically modified mice strains.

1. We used $GR^{LysM/Cre}$ mice with reduced glucocorticoid receptors (GR) on myeloid cells, i.e. on monocytes, macrophages and microglia, to examine the role of glucocorticoid receptor mediated control of cerebral inflammation for stroke outcome. Indeed, $GR^{LysM/Cre}$ mice showed a decreased motor outcome and larger infarcts after stroke, illustrating the importance of glucocorticoid receptor mediated control of cerebral inflammation for stroke outcome.
2. We used mice with impaired glucocorticoid receptor dimerisation (GR^{dim}). GR^{dim} mice do not dimerise the glucocorticoid receptor complexes during receptor activation resulting in a disrupted signal cascade. Stroke survival is dramatically decreased in GR^{dim} and mice compared to wild type indicating a vital role of GR dimerisation in control of glucocorticoid-mediated immunosuppression following stroke.
3. We used mice with eliminated glucocorticoid receptors in neurons to examine to what extent the activity of neuronal glucocorticoid receptors affects stroke outcome ($GR^{CamKII/Cre}$). Glucocorticoid receptor deletion on neurons in $GR^{CamKII/Cre}$ mice is disastrous for stroke outcome suggesting that neuronal regulation of glucocorticoid-mediated immunosuppression following stroke is essential for non-fatal stroke outcome.

Peer reviewed scientific publications

48. Hartmann K, Koenen M, Schauer S, Wittig-Blaich S, Ahmad M, Baschant U, Tuckermann JP. Molecular Actions of Glucocorticoids in Cartilage and Bone During Health, Disease, and Steroid Therapy. Physiol Rev. 2016 Apr;96(2):409-47.

49. Hübner S, Dejager L, Libert C, Tuckermann JP. The glucocorticoid receptor in inflammatory processes: transrepression is not enough. Biol Chem. 2015 Nov;396(11):1223-31.

50. Lim HW, Uhlenhaut NH, Rauch A, Weiner J, Hübner S, Hübner N, Won KJ, Lazar MA, Tuckermann J, Steger DJ. Genomic redistribution of GR monomers and dimers mediates transcriptional response to exogenous glucocorticoid in vivo. Genome Res. 2015 Jun;25(6):836-44.

51. Liu P, Baumgart M, Groth M, Wittmann J, Jäck HM, Platzer M, Tuckermann JP, Baschant U. Dicer ablation in osteoblasts by Runx2 driven cre-loxP recombination affects bone integrity, but not glucocorticoid-induced suppression of bone formation. Sci Rep. 2016 Aug 24;6:32112.

52. Mueller KM, Hartmann K, Kaltenecker D, Vettorazzi S, Bauer M, Mauser L, Amann S, Jall S, Fischer K, Esterbauer H, Müller TD, Tschöp MH, Magnes C, Haybaeck J, Scherer T, Bordag N, Tuckermann JP, Moriggl R. Adipocyte Glucocorticoid Receptor Deficiency Attenuates Aging- and Hfd-Induced Obesity, and Impairs the Feeding-Fasting Transition. Diabetes. 2017 Feb;66(2):272-286.

53. Vettorazzi S, Bode C, Dejager L, Frappart L, Shelest E, Klaßen C, Tasdogan A, Reichardt HM, Libert C, Schneider M, Weih F, Henriette Uhlenhaut N, David JP, Gräler M, Kleiman A, Tuckermann JP. Glucocorticoids limit acute lung inflammation in concert with inflammatory stimuli by induction of SphK1. Nat Commun. 2015 Jul 17;6:7796.

3.5. Development of biomarkers for brain ageing

Summary

Phenotype-related biomarkers of brain structure or global cognitive function provide a close correlation to age at the individual level. Neither immune markers nor telomere length are sufficiently reliable markers to indicate prenatal stress-induced aberrations in brain development ageing.

Brain structural or functional- related markers

One major goal of this part of *BrainAGE* was to determine biomarkers to detect altered trajectories of brain development and ageing in prenatally stressed subjects. The development of markers of brain aging that are related to brain structure (*BrainAge* score, see 2.1) or global cognitive function (cerebral information processing capacity (see 2.2) is well-advanced. These markers show low inter-individual variability and methodological variations of measurements across labs or study sites. The superiority of phenotype-related markers over mechanism-related markers such as the telomere length may be explained on a number of grounds: At present it is easier to determine phenotype because the processes underlying brain aging are complex and not yet well understood. Moreover, the organism modulates and responds to the process of aging in the biological environment through a large variety of compensatory pathways. In contrast to the complexity of pathways at the cellular level, the organism can respond to an infinite number of biological and environmental influences with only limited changes to the phenotype. Consequently, phenotype-related biomarkers based on structural and functional brain development and aging probably better reflect and longitudinally track individual brain aging trajectories.

Metabolic markers

Additional biomarkers that correlate with age comprise metabolic markers. The possibility to broadly scan for metabolic changes or imbalances that are related to age makes metabolomics a very promising method to identify markers of biological age.

Cytokine markers

Cytokines do not represent suitable markers to determine biological brain age or to identify subjects at risk for altered brain development and ageing at the present methodological stage of measurement which is very unreliable for baseline estimations (see 3.4).

Telomere length

Determination of telomere length

Telomeres are non-coding functional DNA repeat sequences at the ends of chromosomes that decrease in length by a predictable amount at each cell division. When the telomeres become critically short, the cell is no longer able to replicate and enters cellular senescence. Therefore telomere length can be a marker of both growth history (cell division) and tissue function (senescence). Accelerated telomere shortening may reflect stress-related oxidative damage to cells and accelerated aging. Severe psychosocial stress has been linked to telomere shortening in some mood disorders that could be observed after severe prenatal stress. Telomere length and percentage of short telomeres were assessed in this project in purified peripheral blood mononuclear cells using hybridization of specific probes (HT Q FISH).

Effects of prenatal stress on telomere length

Maternal anxiety during pregnancy was not associated with leukocyte telomere length and telomerase activity in the offspring at 28- 29y of age (publication in preparation). Similarly, within the Dutch famine birth cohort, leukocyte telomere length and the percentage of short telomeres did not differ at the age of 68y between those exposed to famine during early gestation and those unexposed during gestation (de Rooij, Am J Nutr 2015). However, a lower socioeconomic status at birth, frequent consumption of alcohol (specifically consumption of spirits), a history of cancer, and a lower self-reported health status were significantly associated with shorter leukocyte telomere length. Last but not least, being in employment (job) was significantly associated with a smaller percentage of short telomeres.

3.6. Therapeutic interventions

3.6.1. Interventions during pregnancy

Summary

Improvement of maternal lifestyle before pregnancy has positive effects on offspring brain function which might be at least in part be mediated through normalisation in lipid metabolism.

Nutritional intervention during pregnancy

We conducted a proof-of-concept nutritional intervention study during pregnancy in an overweight women sample from an RCT in which women were randomised to a periconception lifestyle intervention or care as usual. Concentration levels of glycerophospholipids were lower in the intervention group compared to the control group showing an effect on lipid metabolism (Fig. 17). These findings, together with the finding that offspring of women who successfully improved their lifestyle before pregnancy in the intervention arm had fewer behavioural problems suggesting that effects of maternal diets in pregnancy on offspring brain function and behaviour might be at least in part be mediated through alterations in lipid metabolism (publication in preparation).

Metabolite Class	No. of Metabolites
Amino Acids & Biogenic Amines	11
Sugars	1
Glycerophospholipids	46
Sphingolipids	4

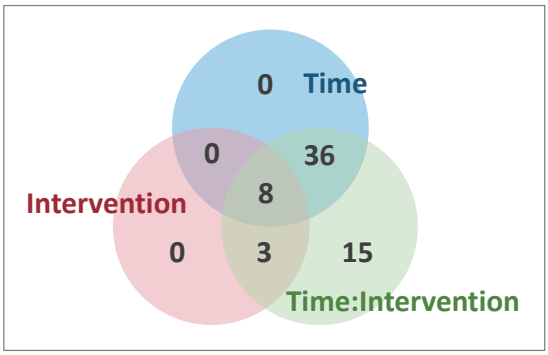


Fig. 17. *Number of metabolites per metabolite class that were significantly affected by the interaction between time and intervention (p<0.05).*

3.6.2. Interventions during later life

Summary

Serotonin re-uptake inhibitors improve stroke outcome in prenatally stressed rats but not in controls when it is administered after stroke. This effect is, at least in part, due to normalisation of increased vasotone. Preventive therapy has no effect and is deleterious in controls.

Serotonin re-uptake inhibitor citalopram

Serotonin re-uptake inhibitors may reverse altered vasoreactivity in prenatally stressed subjects in addition to their anti-inflammatory actions and their ability to reverse altered stress sensitivity by normalising glucocorticoid receptor function. The vasodilatory effect in the cerebral circulation may induce the rare cerebral bleedings described clinically. However, they may also induce positive effects on stroke incidence and outcome when the cerebral vasotone is increased, for example in prenatally stressed subjects. Innovative serotonin re-uptake inhibitors are ideal candidates to administer electively or following stroke because they only induce slight side effects and have few drug interactions.

Daily pre-treatment with citalopram for 4wk before stroke increases infarct volume 28 days after stroke in control rats at middle and old age. The vasodilatory effects of citalopram may result in a higher reperfusion leading to hemorrhagic transformation and an increase of infarct volume. If citalopram is administered for 28 days beginning 2h following stroke, a more clinically relevant approach, infarct volume was not affected. Administration of citalopram probably took place after increased reperfusion of the infarct and, hence, the deleterious effect on infarct volume did not occur.

There was no increase in infarct volume in rats prenatally exposed to dexamethasone if pre-treatment with citalopram begun 4wk before stroke because normalisation of the increased cerebrovascular tone may have occurred. If citalopram was administered beginning 2h following stroke, infarct volume decreased. The vasodilatory effect of citalopram may have improved blood flow into the penumbra and, thus, decreased stroke volume.

4. Potential impact and main dissemination activities and exploitation of results

4.1. Impact

Overall impact

The increased incidence of brain diseases with longevity poses both health and social problems. There is considerable concern at governmental and societal levels that costs relating to the health and social problems of an increasingly ageing society could hugely challenge the European social and health insurance systems in the coming years. The situation calls for pioneering scientific advances which can quickly be translated into preventive strategies and therapy of early brain ageing and brain age-related disorders. In this respect, we have chosen a strong translational approach to investigate the impact of early environmental stimuli on brain ageing.

The prenatal period is increasingly recognized as a target for the primary prevention of diseases in later life. Because maternal life style and stress are modifiable, the study of the prenatal environment in *BrainAGE* has very substantial public health implications in terms of improvement of outcome for children. Knowledge on vulnerable periods and adverse environmental stimuli during pregnancy can be used to raise public awareness.

BrainAGE discovered epigenetic, inflammatory and metabolic mechanisms linking human development and ageing, and translated experimental results (e.g. by using non-human primates) into the human situation. In addition, it provided innovative MRI-based and neurocognitive strategies to detect early brain ageing. Noticeably, *BrainAGE* strongly emphasized gender specific analyses of processes which link human development and ageing. We studied interventions during pregnancy when the effects of prenatal stress are still reversible. Moreover, we successfully explored experimentally approaches to improve increased stroke outcome in prenatally stressed rodents.

The results achieved in the project have shown

(I) the extent to which the different types of prenatal stress program early brain ageing,

(II) that prenatal stress programs structural and functional brain development and early cognitive decline as well as the outcome of stroke in aged rats, and

(III) that epigenetic changes of the glucocorticoid receptor, increased sympathetic activity, and increased cerebrovascular tone are key mediators connecting developmental modifications to early brain ageing and increased susceptibility to age-associated brain disorders.

(IV) The project has identified pharmacological interventions that may reverse altered stress sensitivity and vasoreactivity in rodents as a base for preventive or therapeutic human studies.

Overall, we have obtained a better understanding of the impact of various prenatal stressors on brain development and ageing, and achieved enhanced detection of changes in brain development and ageing induced by prenatal stress. This knowledge enables us to make recommendations to scientists, public health policy makers, and health professionals on how to avoid adverse effects of prenatal stress due to hormone exposure. Our findings will enable policy makers to develop better public health policies, increase public awareness with regard to ameliorating the ageing process and thus, enabling a better integration of an ageing community into society. Thus, *BrainAGE* can provide recommendations to stakeholders and the public relating to the early environmental stimuli that should be avoided at particular times of human development. We inform science policy makers about the importance of the field and suggest areas that merit especial attention.

BrainAGE achieves a prevention effect by education and development of stress reduction instructions. This considerably increases the chance for healthy ageing in future generations.

Preventing early brain ageing also has an impact on reducing brain-related diseases, such as stroke. Stroke is one of the most common causes for disability and invalidism in Europe. Costs for prevention, treatment, rehabilitation and care consume much of the health budget in Europe. These costs rise with increasing life expectancy as stroke is closely associated to age. Thus, having indicators for early brain ageing offers the opportunity for targeted interventions. *BrainAGE* provides both indicators for the diagnosis of early brain age and therapy strategies for intervention that not only benefit the individual but also provide an advantage for the European health budget.

Translating Research for Human Health

The *BrainAGE* consortium brought together and integrated experts from world-leading research groups dedicated to the investigation of the link between human development and ageing. It incorporated unique human cohorts available Europe-wide and – for the translational aspect – non-human primate cohorts based in the US. The consortium has gained leadership in the field of linking human development with brain ageing. We have contributed to a better understanding of the impact of various prenatal stressors, as well as to the improved detection of changes in brain development and ageing induced by prenatal stress.

BrainAGE strongly supported the objectives of the translational research action in the FP7 Health Programme. One of the major problems in science today is the translation of basic research into clinical application. A focus on basic science without such translation into exploitation for the health of the population would not make effective use of essential resources. *BrainAGE* provided the unique opportunity to bundle the expertise of scientists from different nations, fields, and disciplines to generate new knowledge of biological processes and to support the often neglected necessity of translating this knowledge into innovative clinical applications. *BrainAGE* discovered mechanisms linking human development and ageing using molecular biological and genetic approaches, translated experimental results (e.g. by using non-human primates) into the human situation, and provided innovative strategies to detect early brain ageing as well as implement interventional approaches when the condition is still reversible. Another major problem of current research consists in the neglect of gender specificity; often observations obtained in males are used to predict processes in male and female human subjects. *BrainAGE* strongly emphasized gender specific examinations of processes which link human development and ageing.

BrainAGE not only determined in rodents which prenatal stressors have an impact on brain ageing. It also developed markers of brain ageing in a translational approach from rodents via non-human primates into the human situation. *BrainAGE* generated first experimental results of pharmacological interventions in rodents that are ready to be translated for use in non-human primates and in humans. We will exploit these results in the post-project phase.

Linking human development and ageing

Research into human development and ageing is amongst the most important cross-cutting issues in science and society. It is unclear whether an increase in longevity is accompanied by an increase in a disease-free life expectancy, especially in the field of brain-related diseases. We chose a strong translational approach to investigate the impact of early environmental stimuli on brain ageing, supporting the development of relevant early diagnostic and therapeutic approaches when the conditions are still reversible. We bundled state-of-the-art methods and tools available in our consortium to tackle this problem. The standardized examination of our cohorts allows new insights and set new standards in the clinical examination of the effects of early stress on human brain development and brain ageing. Our rigorous and standardised methods also raised the quality of future follow-up examinations of our cohorts to a new level. Considerable added value has been achieved due to the close interaction of scientists who focus on human development with those engaged in the field of ageing on the one hand and between epidemiologists, clinicians and basic scientists on the other hand.

Another focus of *BrainAGE* was to study some of the mechanisms that link human development with ageing. Understanding these mechanisms will create the base for future investigations as well as for the development of preventive measures and effective interventions. Thus, *BrainAGE* will be able to provide direct recommendations at an early stage with regard to which early environmental stimuli should be avoided at what time of human development, and also late (e.g. employed in adulthood or older age) interventional regimes for later stages if babies were already exposed to such prenatal stimuli. This dual value enhances the value of *BrainAGE*; it will considerably improve the health of future generations.

Development and delivery of biomarkers of early brain ageing

One of the major goals of *BrainAGE* was to develop and deliver biomarkers of early brain ageing. Scientific validation and application of these biomarkers beyond national boundaries were also essential elements of *BrainAGE*. Hence, results on biomarkers are of immediate relevance for the ageing society. Healthy brain ageing in the ageing society of Europe is a vital issue for an individual's quality of life and an important issue for the society at large. The diagnosis of early brain ageing using the biomarkers opens the possibility to intervene and support healthy ageing in people at risk.

New findings on biomarkers of ageing were exploited by the SMEs, but also by the scientists involved in *BrainAGE*, e.g. MRI analysis procedures, innovative neuropsychological tests and metabolic markers. The use of the standard T1 MRI sequences to estimate structural brain age by volumetry and the computer-based evaluation of the cerebral information capacity will guarantee a fast exploitation and, thus, access for the European population.

SME participation and innovation

We incorporated two excellent SMEs dedicated to R&D. In addition, they bring into the consortium experience in exploitation and international marketing, ensuring the rapid exploitation of results achieved. For example, metabolic biomarkers to detect early brain ageing will be exploited for innovative diagnostic strategies to aid in the prevention of brain ageing. The SME Biocrates has the experience and potential to develop specialized analytical assays and kits that can be applied in the research and diagnostics of brain age-related diseases. As a partner in the consortium, it is actively involved in the identification of key metabolites for brain ageing, which is the base for innovative and commercially applicable biomarker assays. The knowledge obtained in this project has facilitated ideas and advancement of our and other SMEs. Development of markers of brain ageing has led to new interest in this field and will attract other SMEs to join and continue the work on development of markers for biological age.

By paying attention to the different levels of dissemination (see 3.2), and via exploitation of the results by the SMEs involved in *BrainAGE*, we substantially contributed to the Community Action Programme: Generate and Disseminate Health Knowledge.

Imperative for a European approach - European added value

BrainAge had the unique opportunity to use well-defined existing human cohorts that are not available in any single nation in the world. Bundling state-of-the-art methods and tools available in the consortium and the standardized examination of the cohorts has led to new insights and set new standards in the study and analyses of the effects of early stress on human brain development and brain ageing. Although the expertise of national research groups forms a strong fundament for this project, the European approach exceeded this via comparing human cohorts from different nations, thus allowing conduction of a new quality of research and exchange of knowledge. The project has supported European research in a number of ways. The infrastructure evolved in *BrainAGE* easily allows for opening out and conduction of follow-up studies in our human cohorts. The results from *BrainAGE* offer an attractive platform for pharmaceutical companies to generate additional experimental data by conducting clinical trials on pharmacological interventions. The promotion of healthy ageing comprises a huge market for pharmaceutical companies. An important impact of the European added value is that exchange of knowledge between the participants of

different nations boosts national initiatives and also facilitates the identification of best practice procedures in pregnancy care which currently differ quite strongly in European countries, which will in turn support the development of pan-European health policy strategies.

The possibility for scientists from across the USA (through bilateral agreements with the NIH) to participate in the EU Framework Research Programs will greatly aid in overcoming the dilemma of competition versus cooperation that scientists from Europe and the US are accustomed to. Further, it could also help reduce the number of ambitious European science graduates who leave Europe to work in the US. Funding the collaboration between Europe and the US in *BrainAGE* may serve as an example of competitive research in Europe in cooperation with leading scientists in the US that might motivate and stimulate other scientists to use similar strategies, thus reducing the brain drain from Europe to the US. We anticipate that funds for studying non-human primates will promote an invaluable indirect value in addition to supporting the translational aspect of the project, a fact that would be impossible to achieve with European partners because of the absence of appropriate non-human primate cohorts in Europe

Economic benefits for Europe

Health and other costs associated with early brain ageing include the cost of initial hospitalisation, the cost of further chronic diseases resulting from brain ageing and social costs including loss of earnings of the affected individual and perhaps the loss of employment of the carer as well. The body of evidence that has accumulated indicates that productivity gains and healthy life years gained by optimising prenatal circumstances are both substantial and robust. Preventing early brain ageing also has an impact on reducing brain-related diseases, such as stroke. An improved understand of human biological variation across the lifespan is reached through the multi-level dissemination strategy of *BrainAGE* that is aimed at informing the scientific community, health professionals such as obstetricians and midwifes and the general public (see 3.2). Effective dissemination of the results of *BrainAGE* is supported by the collaborative character of the project at the European level. The different nationalities of the participants support dissemination of the results effectively in different European nations (see 3.2). The participants educate health professionals about the importance of the pre- and postnatal mental health and balanced diet of their patients, a currently often neglected aspect.

4.2. Dissemination

The overall aim of *BrainAGE*'s dissemination activities was and is to ensure a wide-reaching impact and exploitation of project results amongst all stakeholders: scientific peers, media, policy makers and the civil society and funders. The *BrainAGE* Consortium planned the dissemination activities in an iterative manner – by following the outline depicted in the DOW (WP10 Training and Dissemination) and also through meetings (e.g. Kick-off, Symposium) and via discussions in order to identify further activities. The dissemination activities were aligned with key stages of the project and we ensured that the plans offered sufficient flexibility to respond to the needs of the target group as well as towards wider developments in policy and practice. Dissemination was undertaken throughout the duration of the project and will continue in the coming 3-4 years. All project members actively participated in the discussion and planning of the dissemination strategy and contributed to the dissemination activities. The comprehensive and strategic dissemination approach employed by *BrainAGE* has and will ensure a swift dissemination of project results on a European and worldwide scale leading to sustainability of the project results.

The dissemination activities (Tab.2) included organising the first conference on prenatal stress and brain aging and putting together a special issue on this topic in the high impact journal "Neuroscience in Biobehavioural Reviews" (see 4.2) . In this manner, *BrainAGE* has and will continue to stimulate creative research in this field durably and disseminate European scientific and health care excellence internationally.

We presented our results at the two leading international meetings focusing on prenatal development of health and disease in later life (DOHaD World Congress in Cape Town 2015 / Utrecht 2017). During these meetings, we critically discussed our results with public health policy makers, and health professionals such as midwives, obstetricians, geriatricians and family practitioners from both industrial and developing countries.

Taken together, the dissemination activities (Tab. 2) comprised using the traditional communication channels such as events (scientific meetings, organising a symposium and summer school, and workshop), and print media (leaflets, booklets, press releases, conference papers, articles in peer-reviewed journals, posters, flyers etc.), training activities (amongst beneficiaries), the *BrainAGE* consortium also utilised online activities based on the project website. All dissemination activities and publications in the project acknowledged the European Community's Seventh Framework Programme funding. Scientific publications mentioned as far as possible: "This research has received funding from the European Community's Seventh Framework Programme under Grant Agreement No.:279281.

Project period	**Dissemination activities**	**Aims and Target groups**
M1-M6	Creation of logo; creation and launch of website; internal login at website and chat forum: Press release; shared Google documents & Dropbox facilities; Kick-Off Meeting; organize data management for project; plan annual meetings of project beneficiaries;	Achieve visibility at begin of project for all stakeholders e.g. politicians; public health policy makers; institutional academic staff; research groups and the broader scientific community; raise the profiles of participating organisations
M6-M36	Exchange and Alignment of Methods and techniques between beneficiaries; development of scientific protocols and sharing of data; intensify communication between project beneficiaries via email, telephone and video conferencing; organization of Scientific event Symposium "*Prenatal Stress and Brain Disorders in Later Life*; Organization of Summer School "*Prenatal Stress and Brain Disorders in Later Life*"; creation of posters, Flyer and Brochure for Symposium and Summer School; peer-reviewed publications; oral presentations at conferences and other popular topic-related events; articles in popular magazines and press;	Extend and intensify visibility of project and results between beneficiaries and with stakeholders; motivate students and staff to receive training in project topic and field and encourage these to share their knowledge with their peers; Extend visibility to international audience
M37-M60	Continue and intensify all communication and publication activities; plan and coordinate review articles for Special Issue for the Journal Neuroscience and Biobehavioral Reviews; plan for future use of project deliverables e.g. Brain Age Score; method to determine the biological and functional brain age in humans using an innovative neuro-cognitive approach based on a neural theory of visual attention; analysis of project data and preparation and	Increase dissemination, exploitation and sustainability for project results for stakeholders at all levels scientific and society; build network for future collaboration; ensure sustainability of project results

	communication of results;	
M60 - beyond	Set up post-project organisational structure under the leadership of the project coordinator; update the website with new data and results; evaluate achievements and impact; complete publications; develop ideas for future cooperation; work on improved guidelines for medical and public health policies; prepare educational print and online material for obstetricians and midwives; contact relevant media; contacting policy-makers; cooperate with the European Commission by providing useful inputs to its dissemination and exploitation efforts	Share solutions and know how with the scientific community and health professionals; create new opportunities to extend the project and its results and develop new partnerships for the future; influence policy and practice working with policy-makers and health professionals

Tab. 2. *Dissemination activities.*

4.4. Exploitation

One of the main goals of the *BrainAGE* consortium was to study the association between prenatal stress and brain ageing. Over the project period, *BrainAGE* generated innovative methods to determine biological brain age in contrast to chronical age and explored innovative preventive and interventional measures. Through the interdisciplinary efforts of leading European scientists and 2 SME´s, the consortium has gained extraordinary knowledge that lays down the pathway for healthier aging in the future. Use and exploitation of the knowledge generated in *BrainAGE* beyond the lifetime of the project is crucial for the purpose of this project.

The *BrainAGE* consortium will continue to promote and publicize its outcomes beyond the project duration emphasizing the negative impact of different types of stressors during pregnancy on health and behavior of the offspring in later life. The participating SMEs involved in *BrainAGE* (LifeLength / Biocrates) have developed biomarkers of a healthy aging, telomere length analysis and metabolic markers that can be exploited by the scientific community, experimental and public health representatives. Furthermore, the *BrainAGE* consortium will continue to perform research on this topic and to follow the outcome of the cohorts in the future.

The *BrainAGE* consortium has developed a post-project organizational structure under the leadership of the project coordinator. The goal of this post-project structure is to assure that the methods and cohorts will be made available to potential users. The scientists responsible for the human and non-human primate cohorts as well as of the unique rat and mice strains will be available as contacts to potential users. Contact details will be published on the *BrainAGE* website. The knowledge obtained and our dissemination activities have led to new research ideas and a better integration of existing knowledge and resources. The continuous examination of our human unique cohorts and animals is ensured by grant applications from national and international agencies.