Non-invasive biomarkers of fetal brain development reflecting prenatal stress: an integrative multi-scale multi-species perspective on data collection and analysis

Martin G. Frasch¹, Silvia Lobmaier², Tamara Stampalija³, Paula Desplats⁴, María Eugenia Pallarés⁵, Verónica Pastor⁵, Marcela Brocco⁶, Hau-tieng Wu⁷,⁸, Jay Schulkin¹, Christophe Herry⁹, Andrew Seely⁹, Gerlinde A.S. Metz¹⁰, Yoram Louzoun¹¹, Marta Antonelli⁵

¹ Dept. of Obstetrics and Gynecology, University of Washington, Seattle, USA
² Frauenklinik und Poliklinik, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany
³ Unit of Fetal Medicine and Prenatal Diagnosis, Institute for Mother and Child Health IRCCS Burlo Garofolo, Trieste, Italy
⁴ University of California, San Diego, Departments of Neurosciences and Pathology
⁵ Instituto de Biología Celular y Neurociencia “Prof. Eduardo De Robertis”, Facultad de Medicina, Universidad de Buenos Aires, Argentina
⁶ Instituto de Investigaciones Biotecnológicas - Instituto Tecnológico de Chascomús (IIB-INTECH), Universidad Nacional de San Martín - Consejo Nacional de Investigaciones Científicas y Técnicas (UNSAM-CONICET), San Martín, Buenos Aires, Argentina
⁷ Dept. of Mathematics and Dept. of Statistical Science, Duke University, Durham, NC, USA
⁸ Dept. of Mathematics, University of Toronto, Toronto, ON, Canada
⁹ Ottawa Hospital Research Institute, Ottawa, ON, Canada
¹⁰ Canadian Centre for Behavioural Neuroscience, Department of Neuroscience, University of Lethbridge, Lethbridge, Alberta, Canada
¹¹ Bar-Ilan University, Dept. of Applied Mathematics, Israel

Short title: Biomarkers of fetal brain programming

Corresponding author
Martin G. Frasch
Department of Obstetrics and Gynecology
University of Washington
1959 NE Pacific St
Box 356460
Seattle, WA 98195
Phone: +1-206-543-5892
Fax: +1-206-543-3915
Email: mfrasch@uw.edu
Abstract
Prenatal stress (PS) impacts early postnatal behavioural and cognitive development. This process of 'fetal programming' is mediated by the effects of the prenatal experience on the developing hypothalamic-pituitary-adrenal (HPA) axis and autonomic nervous system (ANS). The HPA axis is a dynamic system regulating homeostasis, especially the stress response, and is highly sensitive to adverse early life experiences. We review the evidence for the effects of PS on fetal programming of the HPA axis and the ANS. We derive a multi-scale multi-species approach to devising preclinical and clinical studies to identify early non-invasively available pre- and postnatal biomarkers of these programming effects. Such approach would identify adverse postnatal brain developmental trajectories, a prerequisite for designing therapeutic interventions. The multiple scales include the biomarkers reflecting changes in the brain epigenome, metabolome, microbiome and the ANS activity gauged via an array of advanced non-invasively obtainable properties of fetal heart rate fluctuations. The proposed framework has the potential to reveal mechanistic links between maternal stress during pregnancy and changes across these physiological scales. Such biomarkers may hence be useful as early and non-invasive predictors of neurodevelopmental trajectories influenced by the PS. We conclude that studies into PS effects must be conducted on multiple scales derived from concerted observations in multiple animal models and human cohorts performed in an interactive and iterative manner and deploying machine learning for data synthesis, identification and validation of the best non-invasive biomarkers.
INTRODUCTION

By 2010, 250 million children (43%) younger than 5 years in low-income and middle-income countries are at risk of not reaching their developmental potential. (Lu et al., 2016; Black et al., 2017) This is mostly due to exposure to biological and psychosocial factors that might alter brain function. (Grantham-McGregor et al., 2007; Walker et al., 2007) It is now widely accepted that maternal distress including depression, anxiety, stress, fears and worries have been identified as key risk factors affecting child development that requires urgent intervention. (Walker et al., 2007) (Fontein-Kuipers et al., 2014; Rakers et al., 2017) Early postnatal care was shown to partially reverse the effects of prenatal stress (PS) on brain reprogramming in animal models. (Barros et al., 2004; Weaver et al., 2005; Barros et al., 2006b) Early interventions promoting mother-infant bonding and cognitive stimulation, mainly based on education and maternal support programs and which are accessible in developing countries, might improve developmental outcomes in PS-exposed children, decreasing liability for psychopathology. (Fontein-Kuipers et al., 2014; Nolvi et al., 2016)

PS impacts early behavioral and cognitive development in human infants. (Mulder et al., 2002; Beydoun & Saftlas, 2008) As a result, infants may develop attention-deficit hyperactivity disorder (ADHD) and sleep disturbances. (Weinstock, 1997) Longer-term persistence of these disorders may lead to depression and vulnerability to psychotic disorders in adulthood. (Cohen et al., 1983; van Os & Selten, 1998) The underlying mechanisms of this fetal programming of adult diseases are thought to be mediated by the impact of PS on the developing hypothalamic-pituitary-adrenal (HPA) axis, an essential homeokinetic system capable of responding to stressors. (Van den Hove et al., 2006) HPA axis is highly sensitive to adverse early life experiences (Meaney, 2001). Animal studies show that PS results in vulnerability to anxiety and impaired learning, memory and locomotor dysfunction. (Weinstock, 2001; Huizink et al., 2004) Exposure to PS results in increased responsiveness of the HPA axis to stress, and reductions of glucocorticoid receptor (GR) expression in the hippocampus of adult offspring. (Zuena et al., 2008) In humans, prenatal depressed/stressed maternal mood is associated with higher rates of preterm delivery and lower birth weight, (Wadhwa et al., 1993; Van den Bergh et al., 2005) elevated cortisol, (Field et al., 2004) impaired subsequent working memory performance in young women (Entringer et al., 2009) and changes in the epigenetic regulation of GR expression. (Mulder et al., 1997)

A possible therapeutic avenue to counteract the effects of PS-induced fetal programming is given by postnatal stimulation. This can be accomplished by changes in the postnatal environment, such as care and early adoption. The positive effects of such treatment include better cognitive performance of adult offspring (Meaney et al., 1988) and reduction of stress-induced corticosterone secretion. (Wallen et al., 1999)

The first step in devising and testing early interventions to prevent PS effects on offspring is the identification of changes of the intrauterine environment using reliable and robust biomarkers of stress-related epigenetic reprogramming. We propose a conceptual multi-species multi-scale framework to discover early biomarkers of PS in the exposed infants. First, we review the experimental animal models and human cohort approaches for study of PS. We deliberately focus on two very different animal models: pregnant rat and sheep. The former lends itself to efficient studies of generational effects PS exerts on the offspring. The latter is de facto the only model of fetal physiology that allows fetal instrumentation and chronic monitoring with direct translational relevance to obstetrical practice. We conclude from this section of the review that a combination of such animal models and human cohorts into one systematic multi-species approach holds the key for a comprehensive and clinically relevant modeling of the PS effects. Second, we derive from these observations that such multi-species approach also needs to consider within one paradigm the multiple physiological scales of complexity which all are affected by the PS. Lastly, we review the mathematical instruments which are needed to tackle the complexity of data sets such studies would generate.
PS REWIRES THE BRAIN: A MULTI-SPECIES APPROACH

Various mammalian species have been used to document the multifarious effects of PS on brain development and function ranging form rats to human cohorts. In spite of the wealth of careful retrospective and prospective studies on PS, there are still several confounding factors that cannot be controlled in human studies such as genetic and environmental factors, as well as the social environment. (Weinstock, 2001, 2008) For these reasons, studies in this field continue to rely on animal experiments, due mainly to their shorter life span and short breeding cycles, and because they offer the possibility to control the type, intensity, duration and timing of the stressor applied to the dam, the long term behavioral outcomes, as well as the interaction of the mother with her offspring in a controlled environment. The effects of PS on brain development in animal models have been mainly conducted in Sprague-Dawley, Wistar and Long-Evans rat strains, but also in Rhesus macaques, guinea pigs, sheep and mice. (Amsten, 2000; Kapoor et al., 2008; Weinstock, 2008; Braun et al., 2017)

Most of the prenatal stress rodent models have been performed in rats and these studies have generated a large body of evidence towards the understanding of the mechanisms of developmental programming, especially in relation to the possibility of exploring the brain regions involved in neurogenesis and neuronal plasticity. (Fatima et al., 2017)

Chronically instrumented non-anesthetized fetal sheep is an appropriate and uniquely suited animal model for studying the effects of in utero insults on fetal development, because of its recognized physiological and pathophysiological similarities to human fetal developmental profile and the unique ability to chronically instrument and monitor the fetus while manipulating the intrauterine environment.

Despite the diversity of findings, studies performed in human cohorts have shown the profound impact of PS on the cognitive development of the infants. However, research in humans has mainly been restricted to behavioural studies and to the macroscopic neuroanatomical analyses, while the genetic/epigenetic analysis has been limited to peripheral tissues due to the obvious inaccessibility of the brain. (Braun et al., 2017)

The following will focus on the recent work in rodents, sheep and humans to provide an insight into some of the conceptual framework of mechanisms of perinatal and transgenerational programming.

1) Studies in pregnant rat models of PS

Up to date, the most comprehensive behavioural, morphological and histological information comes from studies in rodent models. (Boersma & Tamashiro, 2015; Weinstock, 2017) For many years, the outcomes were analysed in the first generation offspring only, but more recently a multigenerational paradigm has been established. (Babenko et al., 2015)

a) Single-generational studies

In rodents, various PS protocols have been deployed ranging from saline injections, suspension, crowding, hypoxia, electric foot shock and placental insufficiency to unpredictable stress, noise and REM sleep deprivation. (Huizink et al., 2004; Mastorci et al., 2009) A frequently used protocol is a modified version of Ward and Weisz model consisting of restraining the mothers during the last week of gestation. (Ward & Weisz, 1984) This model induces a robust psychoneuroendocrine stress activation in the mothers. (Mastorci et al., 2009) Abundant evidence demonstrates that exposure to different stressful events during the last week of pregnancy in rats interferes with the physiological progeny development inducing anomalies in neurogenesis and brain morphology that directly affect offspring behavior. (Weinstock, 2001; Mastorci et al., 2009; Charil et al., 2010) PS induces low birth weight, learning and attention deficits, impaired adaptation to stressful conditions, vulnerability to anxiety and depressive-like behaviors, reduced social interaction and some of the characteristic neuronal changes of schizophrenia. (Alonso et al., 1991; Weinstock, 2001; Huizink...
associations between epigenetic regulators that can be modified by environmental factors, such as genetic variations were weak. In the post...

b) Multi-generational studies

The lifetime risk of mental illness is greater if an individual has family history of a similar condition. Therefore, most efforts during the past decades have focused on the identification of genetic associations, but the results were often met with disappointment, as the associations with genetic variations were weak. In the post-genomic era, the focus has shifted to identify associations between epigenetic regulators that can be modified by environmental factors, such...
as stress. Importantly, epigenetic marks are potentially heritable. Trans-generational studies are uniquely suited to reveal mechanisms of trans-generational programming by inheritance of epigenetic, metabolomic and phenotypic traits. The multi-generational stress resembles human populations living in chronic stress conditions, e.g., several generations exposed to residential school, war or poverty. (Laplante et al., 2016) (Santavirta et al., 2017)

Compared to single-generation PS, the multigenerational stress has somewhat different consequences: it facilitates adaptation to the recurrent maternal stress across generations, thus generating stress resilience. This causes new behavioral traits and better brain activity coherence. Based on the mismatch hypothesis, the multigenerational stress leads to better adaptation because the offspring is bred for a stressful environment and the stress is indeed occurring again when the daughters get pregnant. The trans-generational cohort faces the mismatch problem because they are bred for a stressful environment, but there is no more stress during pregnancy or any other time.

1. **PS elevates stress responses and the risk of mental illness.** PS (F1 generation) impedes developmental milestones in rats, thus desynchronizing brain development along with epigenetic signatures of human anxiety, depression, and adverse brain development.(Zucchi et al., 2014) Interestingly, PS-induced anxiety-like and depression-like behaviours become most evident at the most vulnerable periods in life, early development and old age.(Erickson et al., 2014)

2. **PS programs risk of anxiety and depression in future generations.** In a rat model of trans- and multigenerational experience, PS induces increased risk of gestational diabetes, preterm birth, and delayed brain development across generations. These manifestations are linked to microRNA (miRNA) and mRNA signatures of preterm birth(Yao et al., 2014) and mental illness, in particular anxiety and depression-like symptoms, and altered brain connectivity in adulthood.(McCreary et al., 2016) Interestingly, these studies revealed striking sex differences, with stressed females displaying partial stress resilience until the F3 generation, suggesting truly epigenetic inheritance.

3. **Stress-induced epigenetic and metabolic changes propagate across generations.** Stress alters miRNA expression patterns in the brain,(Babenko et al., 2012) thus generating potentially heritable biomarkers of disease. New miRNA pathways have been identified which are involved in neurotrophin and myelin regulation, providing a mechanistic link to mental illness REF. Furthermore, altered epigenetic regulation of gene expression is also accompanied by altered metabolic footprints, which can be assessed in animals and humans using body hair, bio-fluids or solid tissues using NMR spectroscopy and inductively coupled plasma mass spectroscopy.(Ambeskovic et al., 2013) These findings suggest that PS induces stable transgenerational specific epigenetic and metabolic alterations that can also be found in human disease.

MicroRNAs have become appreciated as a means for transgenerational epigenetic inheritance due to their small size, as they can translocate easily during meiosis and fertilization. By contrast, due to the epigenetic mark erasure in the germ line cells, the chromatin remodelling mechanisms represent a more controversial way of transmitting environmental cues across generations. However, there are some reports indicating that certain genomic regions are not demethylated. Thus, they could retain the information to be transmitted to descendants.(Lim & Brunet, 2013) Dias et al. have shown the transmission of odor aversion. They found in the sperm the hypomethylation in the Olfr151 gene that codes a known odor receptor.(Dias & Ressler, 2014) In addition, histone methylation at particular loci in the sperm can be affected by paternal diet and has been associated to an altered cholesterol and lipid metabolism in the offspring.(Carone et al., 2010)
2) Studies in pregnant sheep models of PS

Fetal sheep and guinea pig (Iqbal et al., 2012) in particular have been used extensively for studies of effects of antenatal synthetic glucocorticoid (sGC) treatment on fetal brain development. Many studies showed detrimental acute and transgenerational effects on neurodevelopment and HPA axis responsiveness to stress. (Schwab et al., 2001; McCallum et al., 2008; Antonow-Schlörke et al., 2009; Iqbal et al., 2012; Schwab et al., 2012; Anegroaie et al., 2016) It is not clear whether the postnatal brain can fully compensate for these changes. Consequently, despite the acute benefits of the antenatal sGC treatment to the fetus during labour and in the early postnatal period, further studies are needed to delineate the long-term effects of repeated sGC courses on postnatal brain development. For such fetal/postnatal experimental paradigms, the guinea pig model has been instrumental, although it is possible to do similar work in larger mammals such as sheep or non-human primate. To the extent that sGCs represent a stress stimulus to the fetus and can be injected directly intravenously to the fetus, this experimental approach also represents a possible paradigm for mimicking human fetal stress exposure to maternal stress hormones without accounting for the interindividual and interspecies differences in the placental transfer dynamics. A pharmacological form of PS, antenatal GCs alter the set point of the HPA axis, which matures during late gestation. Taken together, as an iatrogenic stressor or a model for stress-induced fetal programming, sGC-driven studies in guinea pig and sheep have shown the potential of fetal stress exposure to alter organ development, in particular that of the brain. (Moisiasidis & Matthews, 2014b, a)

A more “human-like”, but also technically more complex experimental paradigm involves isolation of pregnant ewes based on the fact that they are flock animals and experience such isolation as stress. Such approach results in acute and chronic stress-induced adaptations and represents the most comprehensive animal experimental model of human fetal stress exposure. (Rakers et al., 2013)

Taken together, these results suggest that PS insults are critical in the development of biochemical responses and behavior in adults, and that maternal care is crucial both during pregnancy and in the first weeks of life. (Fontein-Kuipers et al., 2014) (Nolvi et al., 2016) It has been postulated that several psychiatric disorders that manifest themselves in the adult human, such as schizophrenia, depression, anxiety and drug abuse, are imbalances of dopaminergic, glutamatergic and GABAergic systems as a consequence, among other reasons, of alterations in the early development of the corticostriatal pathway. Rat models of gestational stress will provide clues to understanding the mechanisms by which a PS insult in early life contributes to the breakdown of the balance in neurotransmission and the formation of aberrant cortical connections, which would entail the establishment of abnormal cognitive behaviors.

3) Studies in humans

There is now a large consensus that different types of PS in pregnant women are associated with altered outcomes for the child. Types of stress include maternal anxiety and depression, bereavement, daily hassles, bad relationship with the partner, and exposure to acute man-made or natural disasters. Several independent retrospective and prospective studies (Glover, 2015; Silveira & Manfro, 2015) have shown that PS is associated with lower birthweight and reduced gestational age. (Wadhwa et al., 2011) a poorer performance on the Neonatal Behavioral Assessment Scale, (Rieger et al., 2004) more difficult temperament, (Davis et al., 2007; Werner et al., 2007) sleep problems, (O’Connor et al., 2007) lower cognitive performance and increased fearfulness associated with higher maternal stress during pregnancy. (Bergman et al., 2007)

A well-established cohort is based on the 1998 Quebec Ice Storm which follows the consequences of maternal stress induced by this natural disaster. By studying natural disasters, such as the Project Ice Storm, the impact of maternal objective and subjective distress on
genetic and epigenetic biomarkers can be estimated. (Cao-Lei et al., 2014) Project Ice Storm revealed correlation between exposure to prenatal stress and differential DNA methylation of 957 genes in 13-year old. (Cao-Lei et al., 2016a; Cao-Lei et al., 2016b) The majority of the differentially methylated genes were related to immune function and metabolism. The methylation patterns seemed to mainly correspond to the degree of objective maternal stress rather than subjective stress reported by the mothers during pregnancy.

There are other human cohorts that allowed studies across the generations. In the Överkalix population in northern Sweden, the researchers found a relation between grandparent food availability and the grandchild's longevity. A food excess at ages 9-12 years of grandfathers correlated with short survival of grandsons. These effects might be triggered by methylation of epigenetic marks. (Bygren et al., 2001) Famines during the first and second war (Germany, 1916-18 (Van den Berg & Pinger, 2014) and Amsterdam, 1944-45 (Roseboom et al., 2001) also showed that exposure to adverse environment during the early developmental stages changes the outcomes of the next generations.

In sum, there is large body of evidence for the complexity of PS effects on the individual's physiology and the heterogeneity in the stress responses. Because of the interplay between genes and environment, finding PS biomarkers requires a multispecies approach. Rodent models serve to obtain single and transgenerational markers, sheep fetuses resemble closer human physiology and allow in utero monitoring while the human cohorts allow to analyze the reliability of the putative biomarkers.

A MULTI-SCALE APPROACH TO DISCOVERY OF BIOMARKERS AND TREATMENT STRATEGIES

Epigenetic markers may be correlated with maternal stress, depression and anxiety and with infants’ cognitive development thus serving as novel biomarkers of PS. The animal model-centered review of the PS effects points to the impact of the PS on multiple physiological scales, from molecular level to complex system’s level patterns. This dictates that to derive meaningful, translational biomarkers from preclinical and clinical studies, PS effects should be studied on those molecular and integrative levels in a unified multi-scale paradigm. Such unification can be achieved when multiple pertinent animal models and human cohorts studied are designed in concert, rather than as separate studies. In following we review the physiological scales relevant to gauging the PS effects comprehensively. We propose that such approach will yield clinically relevant PS biomarkers. Aside of biomarkers discovery, studying all physiological scales combined holds the potential to provide insights into therapeutic interventions to recover the PS brain phenotype. Such multi-scale paradigm requires novel mathematical methods of pattern discovery and integration. As we conclude below, rapid developments in machine learning hold the key to this methodology. (Marschik et al., 2017)

1) PS influences brain development epigenetically

Studies on single, multi and transgenerational stress inheritance mechanisms have been conducted mostly using pregnant rat model of inescapable stress. (Monteleone et al., 2014; Yao et al., 2014) Other studies (reviewed in (Ho & Burggren, 2010; Blaze & Roth, 2015)) used maternal separation (Pusalkar et al., 2016) or alterations in maternal behavior (Weaver et al., 2004) or diet (Berardino et al., 2017) as early-life stressors and found changes in DNA methylation, histone modifications and microRNA expression.

We propose that PS may result in patterns of co-variation between DNA methylation and levels of microRNA between brain, blood and saliva in rodent and sheep models of PS. This would serve as a model to validate saliva as the clinically easily accessible peripheral fluid serving as a biomarker of PS exposure and to correlate methylation levels with behavioral outcomes and stress responsiveness.
Epigenetic changes can persistently alter gene transcription affecting physiology and behavior and are thought to underlie these long-term effects of PS. (Weaver et al., 2004; Caldji et al., 2011; Mulligan et al., 2012) Increased HPA stress reactivity in the offspring of low maternal care rats is associated with higher DNA methylation at the promoter of NR3C1 (which encodes GR). (Liu et al., 1997; Francis et al., 1999; Weaver et al., 2004) More recently, Braithwaite et al., (2015) reported that maternal prenatal depressive symptoms significantly predicted increased NR3C1 1F DNA methylation in buccal cells of male infants. (Braithwaite et al., 2015) In mice, levels of both OGT (O-linked-N-acetylglucosamine (O-GlcNAc) transferase) and its biochemical mark, O-GlcNAcylation, were significantly lower in males and further reduced by prenatal stress. (Howerton et al., 2013) In humans, differential methylation is associated with prenatal exposure to maternal depression. (O’Connor et al., 2003; Teh et al., 2014) PS and birth weight. (Filiberto et al., 2011; Mulligan et al., 2012; Vidal et al., 2014)

Preconceptual or intra-gestational stress may result in increased cerebral and placental expressions of the corticotropin-releasing hormone (CRH) gene stimulating fetal cortisol and adrenocorticotropic hormone (ACTH) and signaling premature maturation of fetal tissue. (Horan et al., 2000; Moog et al., 2016) Repeated stress exposure may dysregulate HPA axis and increase CRH and cortisol levels which in turn sensitizes women to stress experienced during pregnancy. Pre-gestational stress increased the expression of corticotropin-releasing hormone type 1 (CRH1) messenger RNA in the brains of mothers and offspring, suggesting an epigenetic route of transgenerational transmission. (Zaidan et al., 2013) Pre-gestational stress to female rats two weeks prior to mating resulted in reduced anxiety, enhanced fear learning, and improved adaptive learning for second generation offspring. (Zaidan & Gaisler-Salomon, 2015) Levels of the stress hormone corticosterone (an indicator of HPA axis functioning) were altered across the three generations in a sex-dependent manner. (Zaidan & Gaisler-Salomon, 2015) Maternal stress during the third, but not the second, week of gestation in rats was associated with alterations in stress reactivity behaviors and prolonged elevations in glucocorticoid levels among adult male offspring. (Koenig et al., 2005) Heightened anxiety was associated with greater CRH mRNA gene expression in the amygdala, and attenuated stress responses were associated with greater glucocorticoid mRNA expression in the hippocampus and impaired feedback to the HPA axis. (Grundwald & Brunton, 2015) The offspring of rats exposed to either a daily injection of corticosterone or prenatal stress during the third week of gestation all displayed decreased GR protein levels in the medial prefrontal cortex, hippocampus, and hypothalamus, as compared to controls. (Bingham et al., 2013) Similarly to the pre-gestational dysregulatory effects of stress on responsiveness to stress during pregnancy, increased CRH levels during stressful pregnancy act on the CRH receptor 1 (CRH-R1) to mediate increased maternal vulnerability after delivery with a suppressed HPA axis increasing the risk for postpartum depression. (Meltzer-Brody et al., 2011; Engineer et al., 2013) Overall, changes in maternal and offspring HPA axis function are modified via stress-induced changes to CRH expression (Zaidan et al., 2013) and often accompanied by behavioral effects.

The studies focused on the HPA-axis have overlooked other physiological system affected by PS, such as the autonomic nervous system (ANS). In fact, (Bleker et al., 2017) suggested that psychosocial stress in pregnancy might program the fetus through other mechanisms than through altering maternal cortisol levels. Moreover, (Braithwaite et al., 2015) found no association between maternal cortisol and infant DNA methylation suggesting that the effects of maternal depression may not be mediated directly by glucocorticoids; instead, sympathetic nervous system activity, a component of the fetal ANS, may be the mediating pathway.

The following genes have been implicated in the stress response in relation to the HPA axis (paragraphs A,B,C,D) and to the ANS (E,F,G).
A) exon 17 of nr3c1 encodes the glucocorticoid receptor (GR), which can function both as a transcription factor that binds to glucocorticoid response elements (GRE sites) in the promoters of glucocorticoid responsive genes to activate their transcription and as a regulator of other transcription factors. It has been associated to a lower GR expression in hippocampus and with an exacerbated response to stress. (Weaver et al., 2004; Murgatroyd et al., 2009; Hackman et al., 2010; Kertes et al., 2016)

B) Intron 1 of FKBP5 that contains GRE sites. The protein FKBP51 belongs to the immunophilin protein family, which plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking. FKBP51 functions as a co-chaperone that interacts with the GR protein. Chronically-administered glucocorticoids reduce methylation at FKBP5 locus. (Lee et al., 2010) In humans, war trauma modified methylation of this gene. (Kertes et al., 2016)

C) HSD11B2 encodes the enzyme corticosteroid 11-beta-dehydrogenase, a microsomal enzyme complex responsible for the interconversion of cortisol and cortisone.

D) CRH encodes the corticotrophin releasing hormone (CRH), one of the major HPA regulators. Changes in CRH methylation have been associated with chronic stress in animal models (Mueller & Bale, 2008) and in humans. (Kertes et al., 2016)

E) GNAS1 encodes the alpha-subunit of the G-protein. This GNAS1 subunit has been associated with ANS (Yasuda et al., 2004) and, more recently, with prenatal maternal stress. (Vangeel et al., 2015)

F) ELP1/IKBKAP encodes a scaffold protein and a regulator for three different kinases involved in proinflammatory signaling. This protein can bind NF-kappa-B-inducing kinase (NIK) and IKKs through separate domains and assemble them into an active kinase complex. Mutations in this gene have been associated with familial dysautonomia. (Jackson et al., 2014)

G) IGF2: this a member of the insulin family of growth factors, involved in development and growth. An association was reported between DNA methylation in one IGF2 DRM and pregnancy-related anxiety. (Vangeel et al., 2015)

b) PS inheritance via miRNA signaling

Experimental paradigms of PS in animal or human cohorts allow for blood sampling and extraction of T cells and PBMCs to analyze microRNA profiles from cellular RNA. These microRNA patterns can be compared to those measured in animal studies yielding data from the relevant brain structures such as prefrontal cortex and dentate gyrus of the hippocampus. PS modified the expression of several microRNAs in the hippocampus and prefrontal cortex of prepubertal and adult rat offspring, microRNA-133b being altered most significantly. (Monteleone et al., 2014)

In animal studies, it is important to account for microRNA spatial expression variation and co-localization. microRNA targets of interest are those related to HPA axis function such as GR, MR, 11b-HSD type 1 and type 2, FKBP5, STAT5B and MHC II, Hsp70 and Hsp90, and to neuronal plasticity and psychopathologies, such as cortical BDNF and glial cell-derived neurotrophic factor (GDNF). Previous analyses have shown that differentially regulated microRNAs included miR-34 (anxiety), and miR-132, 142-5p, 146a, 181b, 486-5p, 650 (depression), miR-124 (regulates GR expression) (Babenko et al., 2015) and the miR-200 family.

2) PS and metabolome

Even mild maternal stress induces epigenetic and metabolomic alterations across four subsequent generations of rats. Notably, many of the epigenetic and metabolic signatures altered by transgenerational stress in this rat model have been also identified as markers of mental illness in humans. (Zucchi et al., 2014)
Future studies can expand these findings with deep sequencing and $^1$H nuclear magnetic resonance (NMR) spectroscopy to identify DNA methylation and microRNA signatures linked to impaired mental health using blood across species such as human, fetal sheep and rat cohorts and link epigenetic and metabolomic profiles to endocrine markers of elevated stress response and adverse mental health outcomes. The multi-species approach would allow to search for similarities between metabolomic patterns to identify possibly predictive epigenetic and metabolomic signatures of PS and transgenerational inheritance that are phylogenetically preserved. Corresponding epigenetic, genetic, behavioral and pathophysiological data can then be correlated with metabolomic outcomes. Determining metabolic linkages to brain development, mental health and wellness outcomes throughout the life-span and across generations has the potential to revolutionize the future of health care by transforming the current trends of curative care to personalized and preventive medicine.

We speculate that prenatal and transgenerational stress, through altered epigenetic regulation, programs the maternal, infant and child stress response and lifetime mental health trajectories. We predict that stress response and mental health status will be associated with distinct metabolic signatures in clinically accessible tissues such as saliva or placenta.

3) PS and microbiome

A surprising recent result that may help understand PS and perhaps even allow monitoring it involved the gut microbiome. The discovery of the placental microbiome re-fueled the debate whether fetus is exposed to and interacts with bacteria during development.(Aagaard et al., 2014) It remains controversial whether the fetal compartment is colonized,(Boersma & Tamashiro, 2015) but the notion continues to attract attention, because such physiological mechanism would have profound impact on brain-gut communication via the vagus nerve(Liu et al., 2015) hence influencing the fetal brain development.(Garzoni et al., 2013; Leclercq et al., 2017) Gut microbiota are essential to human health, playing a major role in the bidirectional communication between gut and brain.(Borre et al., 2014; Haberman et al., 2014) The significance and influence of the fetal intestinal microbiome on stress responses, epigenetic modifications and brain development remain to be explored. Interactions between the microbial community and the developing brain likely contribute to pathological brain development after birth.(Borre et al., 2014; Haberman et al., 2014) Future studies will test PS effects on microbiomes of fetal gut and placenta in animal studies and in human placenta (clinical cohorts) to derive predictive biomarkers of PS.

4) PS and ANS

Most PS-induced alterations have been described for hippocampal and prefrontal cortex neurons.(Fujioka et al., 2006; Negron-Oyarzo et al., 2015) However, changes in the morphology and the connectivity of the autonomic nervous system (ANS) neurons due to PS have been poorly studied. Like the cortical neurons, those from the ANS may also be affected during PS exposure. In the guinea pig, it has been observed that enteric neurons (i.e., peripheral ANS neurons) respond to CRH.(Liu et al., 2005) Patients suffering from panic disorder provide a clinical model of stress. These patients show changes in the sympathetic nervous system also observed in patients with essential hypertension. A reduced neuronal noradrenaline reuptake is present in both disorders and epigenetic changes mediate them.(Esler et al., 2006)

During pregnancy, two lines of investigations have indicated a role of the ANS in mediation of stress effects on fetal physiology and development.

First, maternal corticosteroid administration during pregnancy - frequently used for fetal lung maturation in cases of threatening preterm delivery and an iatrogenically administered pharmacological stressor - has shown to affect autonomic balance in utero.(Dawes et al., 1994; Derks et al., 1995; Mulder et al., 1997; Senat et al., 1998) This effect is transient, but repeated fetal administration of betamethasone alters nervous system maturation.
Second, the vagus nerve influences brain function and body metabolism in a pleiotropic manner. (Pavlov & Tracey, 2012, 2015) A new field of bioelectronic medicine is emerging. It aims to devise therapeutic approaches using vagus nerve stimulation (VNS) to modify the endogenous salutary signaling of the vagus nerve. (Borovikova et al., 2000; Kwan et al., 2016; Pavlov & Tracey, 2017) VNS reduced sympathetic tone, stress-induced anxiety behaviors and depression symptoms in animal models and in clinical studies. (O’Keane et al., 2005; George et al., 2008; Caliskan & Albrecht, 2013; Liu et al., 2013; Clancy et al., 2014; Pena et al., 2014; Ylikoski et al., 2017) VNS is thought to facilitate tonic inhibition of the basolateral amygdala by the infralimbic region of the medial prefrontal cortex, which results in reduced fear response. (Caliskan & Albrecht, 2013) VNS increases CRH expression in hypothalamus (Hosoi et al., 2000) and CRH receptor 1 agonism increases vagal modulation of HRV. (Porges, 1995; Farrokhi et al., 2007; Porges, 2009) This reciprocal CRH - vagus nerve circuitry provides an important diagnostic and therapeutic link between stress and the ANS.

Notably, novel non-invasive methods of VNS are being developed which will not require surgical cervical VNS implants, have minimal to no side effects, and are low-cost. (Liu et al., 2013; Clancy et al., 2014; Frangos et al., 2015; Ylikoski et al., 2017) It is now possible to conceive of VNS treatment of neonates.

Together, there is strong evidence that vagus nerve activity is a key player in PS-induced brain programming, can be monitored using innovative fetal heart rate (FHR) analysis techniques (reviewed in detail below) and used as endogenous homeostatic mechanism to potentially recover the PS induced phenotype early postnatally. This offers another pillar of interventions, complementary to the neurobehavioural strategies such as enrichment.

Fetal ANS function can be studied longitudinally using FHR analyses to measure biomarkers of PS-induced epigenetic reprogramming in human fetuses. Mothers identified as having been “stressed” and controls can be monitored with trans-abdominal non-invasive fetal and maternal ECG (ta-fECG; ta-mECG) for ANS assessment. Infants’ cognitive development can be assessed by the Bayley Scale III of Infant development at 18 months of age. This approach also permits quantification and correlation of ANS and behavioral data to the epigenetic biomarkers from salivary DNA obtained from the neonates and young infants.

Advanced FHR monitoring techniques such as phase-rectified signal averaging (PRSA) or multidimensional FHR analysis are sensitive to detecting an impairment of fetal ANS. (Huhn et al., 2011; Graatsma et al., 2012; Lobmaier et al., 2012; Casati et al., 2014; Frasch et al., 2014; Rivolta et al., 2014; Li et al., 2015; Stampalija et al., 2015) Future studies will test their ability to identify fetuses affected by PS.

Advanced analysis of FHR patterns specifically assessing changes in the autonomic regulation of FHR may identify fetus at increased risk for pathological fetal programming. Early signs of hypoxemia are found in changes in the autonomic regulation of the FHR. This can be assessed by the relatively new PRSA method measured by cardiotocography (CTG) or electrocardiography (ECG) (Bauer et al., 2006; Kantelhardt et al., 2007) even in fetuses. (Stampalija et al., 2016)

a) Phase-rectified signal averaging method

Initially, PRSA has been described in adult cardiology for prediction of mortality after myocardial infarction and has been found to be superior to other methods. (Bauer et al., 2006) PRSA can eliminate signal artifacts and noise and extract areas of interest. In contrast to other methods of analysis of FHR variability, PRSA permits the detection of quasi-periodicities in non-stationary data. PRSA has been successfully applied in fetal medicine, despite the challenges of a non-stationary signal, with more disturbance in the signal than in the adult after a myocardial infarction. The novel parameter referred to as cardiac average acceleration and deceleration capacity is more specific than the conventional FHR analyses (e.g. computerized cardiotocography and short term variation) in identifying intrauterine growth restriction (IUGR).
antepartum (Huhn et al., 2011; Graatsma et al., 2012; Lobmaier et al., 2012; Lobmaier et al., 2016) and strongly correlates with acid-base biomarkers during acute hypoxic stress in humans during labour (Georgieva et al., 2014) and the fetal sheep model (Rivolta et al., 2014) Even more interestingly, it has been shown that IUGR fetuses with brain sparing (fetal adaptive mechanism to chronic hypoxemia) have a lower acceleration and deceleration capacities than growth restricted foetuses without brain sparing (Stampalija et al., 2016) This intimate inter-relation between brain perfusion and FHR is thought to be mediated via ANS (aortic chemoreceptors and carotid baro- and chemoreceptors). Newer data also show an activation of ANS in fetuses affected by maternal gestational diabetes which could not be seen using conventional techniques (Lobmaier et al., 2017)

To evaluate the ANS influence on FHR the beat-to-beat information (R-R intervals) should be analysed. The new generation of the trans-abdominal fetal ECG monitors (such as Monica AN24, Monica Healthcare, Nottingham, UK) allow for a completely non-invasive and passive recording of fetal and maternal ECG: it only records electrophysiological signals from the women’s abdomen without hampering mobility or other diagnostic procedures (Stampalija et al., 2012) This signal can be then used for a more sophisticated analysis of FHR such as PRSA or the multidimensional FHR analysis.

b) Maternal-fetal heart rate entrainment and multidimensional FHR variability analysis in fetal sheep and human cohorts

Although the evidence of maternal-fetal heart rate entrainment, also referred to as synchronization, has been demonstrated (Van Leeuwen et al., 2009) its clinical potential as easily obtainable diagnostic or prognostic tool has remained untapped. We propose that both approaches should be explored both in large animal models and clinical studies to test their potential to predict maternal and fetal stress.

Complex signals bioinformatics approaches have been recently developed (Herry et al., 2016) that will allow examination of the putative correlations between the measures derived from all heart rate analyses techniques and epigenetic markers, based on the assumption that PS imprints both phenotypic modalities permitting a mutual inference.

HRV analysis can be performed via a series of automated algorithms that process a waveform recording into a comprehensive multivariate characterization of its degree of variability and complexity (See for example, the Continuous Individualized Multiorgan Variability Analysis (CIMVA) software engine, Fig. 3) (Seely & Newman, 2016) First, individual heartbeats are identified from the ECG waveform, using commonly used QRS delineation algorithms a time series of R-peak to R-peak time intervals (RRI) is formed. A thorough automated assessment is performed on the quality of the ECG signal and RRI time series. Movement artefacts, noise, disconnections and saturations are identified on the ECG waveform. A beat-by-beat signal quality index can be derived, using continuity and morphology analyses. In addition, the RRI time series is filtered to exclude or correct non-sinus beats and non-physiologically plausible data. The signal complexity and degree of variability are then assessed using the cleaned RRI time series.

FHR variability monitoring requires tracking HRV over time and a moving window analysis is typically employed, whereby a window of fixed duration (or fixed number of RR intervals) is shifted in time across the entire duration of the RRI time series. A comprehensive set of linear and nonlinear variability metrics are calculated within each window, as each technique provides a unique perspective on the data and no single method can provide a complete characterization of the biologic signals. Rather, a combination of multiple techniques stands to deliver the most complete evaluation (Table 1; for detailed description of the HRV measures see Table 2) (Goldberger et al., 2002; Bravi et al., 2011) Variability metrics include measures characterizing the statistical properties (e.g. standard deviation, RMSSD), the informational complexity (e.g. entropy measures), the pattern of variations across time scales
(e.g. fractal measures, power law exponents) or the energy contained in the signal (e.g. spectral measures). Only high quality variability estimates are used in subsequent modelling. The output of the FHR variability analysis is a multivariate representation of variability tracked over time, where the temporal relation between subsets of fHRV measures can help characterize the fetal innate immune system’s response to endotoxin and monitor fetal inflammatory response. For example, in a fetal sheep model of inflammation Durosier et al. (Durosier et al., 2015) calculated a large set of fHRV measures to track variability changes and the impact of LPS injection and resulting inflammation over time. Using population-based Principal Component Analysis (PCA) derived from LPS-injected animals, animal-specific fetal HRV temporal profiles were created, which tracked pro-inflammatory cytokine IL-6 profiles (Fig. 2).

In the fetal sheep model, FHR variability reflects maturation and activation of the parasympathetic branch of the ANS involved in sensing and control of fetal acidemia, hypoxia and inflammation. (Frasch et al., 2007; Frasch et al., 2009; Durosier et al., 2013) In human cohorts and in the fetal sheep model of human labor and fetal inflammation, multidimensional FHR variability analysis can predict clinical outcomes after birth.(Liu et al., 1997; Durosier et al., 2013) In elephants, a highly complex social species with brains similar to humans, HRV-based techniques have been suggested to distinguish stressed versus non-stressed animals. (Vezeina-Audette et al., 2016) To build such predictive FHR acquisition systems, certain types of FHR monitors are required, such as the AN24 monitor. Such monitors have the advantage over traditional ultrasound now used for FHR monitoring in that they sample FHR at a frequency high enough (900 Hz) to enable detection of the more subtle fluctuations of FHR variability which reflect integrative pathophysiological fetal responses such as those to acidemia and likely also to stress. (Durosier et al., 2014; Frasch et al., 2014)

MACHINE LEARNING APPROACHES

The advance of technology allows us more convenient ways to observe the world, and accumulate more data from the world. The relationship between PS and how it impacts early postnatal behavioural and cognitive development is not an exception. We could now easily collect data, which could be direct or indirect measurements of the PS, for example, the DNA methylation, metabolome, microbiome, and ANS activity. While collecting data is relatively easy, without a proper data analysis, we cannot gain any benefit from the data. Machine learning is a fancy name for statistical tools aiming at such a data analysis (Friedman et al., 2001). Conversely, to properly apply these tools, understanding the data is inevitable. The available data for PS have at least the following properties: data are collected from multimodal equipment and are of heterogeneous types, the system under observation for the PS is nonlinear and nonstationary, the data volume could be large and inconsistent across different facilities. Moreover, data quality could vary greatly and data collection velocity could be unpredictable. Based on these facts, choosing proper machine learning tools that are accurate in the prediction, stable to noise and computationally affordable could be challenging. New tools might need to be developed. We summarize an overall machine learning framework in Figure 4.

There are two main steps in machine learning: feature extraction and learning/regression. While there are several measurements available from different aspects for the PS, designing and selecting proper features from these measurements (Guyon & Elisseeff, 2003) is the key step toward successful machine learning. A typical challenge is the combination of heterogeneous data types, such as time-series, imaging, microbiome gene expression profile (OTU frequencies), and blood biomarkers. New learning methods will be required to develop multi-modal learning algorithms. Based on the needs to capture the nonlinearity and nonstationarity, a manifold learning technique called alternating diffusion, based on the low-dimensional geometric structure assumption, has been shown to be useful in fusing information from different modalities in the nonlinear fashion. It allows us to preserve the nonlinear/nonstationary underlying structure and remove the sensor-specific unwanted
nuisance, before the learning procedure is applied (see, for example (Talmon & Wu, (2017))). Another typical challenge is the dimension reduction: as the data gets complicated and measurement gets more diverse, we need a more sophisticated way to identify those useful parameters/features and guarantee numerical. In addition to the traditional linear approaches as the principal component analysis, several nonlinear tools have been developed for this purpose, such as the locally linear embedding (Roweis & Saul, 2000), ISOMAP (Tenenbaum et al., 2000), diffusion map (Coifman et al., 2005), and so on. Sometimes this kind of dimensional reduction algorithm is called unsupervised learning. There are many learning techniques available, ranging from the traditional linear/logistic regression to the modern support vector machine, boosting and random forest, etc. (Friedman et al., 2001) We call these learning techniques the supervised learning, which means that we are learning the system based on the experts' labeling or truth. How to choose a proper learning tool depends on the knowledge of the target problem.

A recently active research field in machine learning is the deep learning framework, which in brief is a generalization of the single layer neural network framework to multiple layers (and hence the nomination deep). (LeCun et al., 2015) One main feature of deep learning is the ability to combine the feature selection and learning steps in a unified framework. However, up to now, there is little theoretical understanding of how it works. How to design an efficient neural network topology for a given problem still remains an art, and a lot of trial and error and ad hoc experience are needed. Moreover, without theoretical understanding, it might be difficult to derive physiological insights from the established neural network, even if it provides a powerful prediction accuracy. Also, while no theoretical guarantee, based on practical experience, it might take a lot of high quality data to make it work properly, which might limit its application to the clinical settings. Despite its theoretical limitations, such deep learning approach has obtained many successes in practical problems. Many variations have been developed in many domains, for example, algorithms to combine audio and video, or the recognition of emotion from multiple modalities,(Mroueh et al., 2015; Kahou et al., 2016) and using auto-encoders limiting the dimension of most inputs,(Said et al., 2017) to name but a few.

While it is possible to learn features from the collected raw data by designing a suitable deep neural network, in the clinical setting it might be beneficial to combine the above-mentioned machine learning techniques in different ways. For example, unsupervised learning techniques could help extract intrinsic genuine features out of the raw data, and allow the deep neural network to focus on the feature organization for the prediction purpose. By doing so, we might render the machine learning framework more interpretable, and might reduce the number of cases needed for the deep learning. Another example of combining supervised and unsupervised learning methods is that in multiple studies of young infants time series (Feldman et al., 2011; Weisman et al., 2011), where it has been shown that neonatal behavior can predict trajectories of neurobehavioral, emotional, and cognitive growth. (Weisman et al., 2011) The effect of early childhood conditions on adult behavior can be translated through time series analysis and machine learning into clear prediction of the adult state, based on the observed neonatal features (Fig. 4). On the other hand, in the clinical setup, we may consider a multi-stage approach (e.g. (Basu Roy et al., 2015)): first, high-risk candidate will be detected using standard tests (e.g. microbiome or FHR monitoring). For high-risk candidate, more complex measures, such as metabolome, will be combined with the microbiome and other methods to produce better classifiers. (Larsen & Dai, 2015) In brief, although it is desirable to have a unified universal framework suitable for analyzing versatile medical data, particularly those for the PS, in practice a careful design of the machine learning framework based on the physiological knowledge and clinical setup is needed.
OUTLOOK

As evidenced in the following presentation of the literature, multiple preclinical studies in rodent and fetal sheep models of prenatal stress can now be translated into clinical studies involving pregnant mothers and infants. Methylation and microRNA levels can be correlated with maternal stress, depression and anxiety and with infant’s cognitive development to assess the sensitivity of this novel biomarker. Similar data now begin to emerge for histone acetylation in the offspring as a function of maternal stress. We propose that increased stress, depression, and anxiety will have a positive relationship with increased methylation and distinct signatures of histone acetylation. Similarly, there is a growing body of literature on clinical studies validating the advanced FHR techniques for example to detect or predict fetal chronic hypoxia or acidemia at birth. This approach can now be extended to test if these monitoring techniques are useful for detecting PS. We attempted a visualization of the complex multi-scale PS or healthy phenotype in Figure 5.

By integrating multiple non-invasively obtainable sources of information using novel epigenetic, electrophysiological and biochemical approaches via machine learning techniques, the proposed framework could yield progress in maternal–fetal medicine, offering a more precise and truly personalized prediction and new possibilities for designing interventions to improve neurodevelopmental outcomes of pregnancy affected by PS.
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Figure legends

**Figure 1.** A schematic representation of the dopaminergic pathways in the rat brain. Dopaminergic neurons can be divided into four groups: nigrostratial, mesolimbic, mesocortical, and tuberohypophyseal systems (see text for details). b Schematic representation of the alterations in the dopaminergic system in the adult rat brain of prenatally restrained stressed rat males. Note that impairments of the dopaminergic system are observed mainly in limbic areas of prenatally stressed rats. From Baier et al, 2012.

**Figure 2.** Temporal profile of a fetal HRV composite measure (principal component analysis, PCA) tracks accurately the pro-inflammatory cytokine IL-6 (pink bars). The red line represents the PCA for IL-6 deviations from baseline in response to intravenous LPS-injection at 0 h (LPS, n = 10 fetal lambs); LPS is the immune stimulus from gram negative bacteria (“infection”). The green line represents the PCA for IL-6 deviations from baseline for control (saline-injected) animals (CON, n = 7). Lightly shaded areas correspond to the confidence intervals around the mean. The baseline value is represented by the dotted blue line.

**Figure 3.** Analytical flow for the derivation of a heart rate variability time series.

**Figure 4.** Proposed framework for future studies of PS effects in a hybrid animal/human experimental design to accelerate biomarker discovery and validation. Note the multi-scale approach spanning several species and data acquisition techniques with varying spatio-temporal resolution. This requires machine learning techniques to derive at risk assessment algorithms for detection of PS exposure and prediction of neurodevelopmental outcomes.

**Figure 5.** A complex multi-scale phenotype of the healthy or prenatally stressed individual. Can the rapidly advancing machine learning techniques help distinguish such individual phenotypes based on all its features across the scales of observations, from microbiome, over to epigenetic landscape to the heart rate (HR) time series?
Table 1. Description of heart rate variability domains. *

| Domain     | Features                                                                                                                                                                                                 |
|------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Statistical| The statistical domain consists of statistical measures (mean, standard deviation, Gaussian, and so on) describing the data distribution. It assumes the data originates from a stochastic process. |
| Geometric  | The geometric domain describes the properties related to the shape of the dataset in space. This includes, in a deterministic system, grid counting, heart rate turbulence, spatial filling index, and Poincaré and recurrence plots. |
| Energetic  | The energetic domain describes the features related to the energy or the power of the data, such as frequency, periodicity, and irreversibility in time.                                                        |
| Informational | The informational domain describes the degree of complexity and irregularity in the elements of a time series, such as distance from periodicity or from a reference model. It includes various measures of entropy (compression, fuzzy, multiscale, and so on). |
| Invariant  | The invariant domain describes the properties of a system that demonstrate fractality or other attributes that do not change over either space or time. Included are scaling exponents, fluctuation analysis, and multifractal exponents. |

* Domains suggested for continuous individualized multiorgan variability analysis (CIMVA platform). Reproduced with permission from Durosier et al., 2015.
Table 2. Measures included in each domain of fetal heart rate variability for continuous individualized multiorgan variability analysis.

| Domain       | Fetal heart rate variability measure                                                                 |
|--------------|------------------------------------------------------------------------------------------------------|
| **Statistical** | Coefficient of variation (based on intervals)                                                        |
|              | Form factor                                                                                          |
|              | Interquartile range                                                                                  |
|              | Kurtosis                                                                                             |
|              | Lee parameter                                                                                        |
|              | Mean value                                                                                            |
|              | Mean rate                                                                                            |
|              | Mean of the differences                                                                              |
|              | Root mean square of successive differences of R-R intervals                                           |
|              | Skewness                                                                                             |
|              | Standard deviation                                                                                   |
|              | Standard deviation of the differences                                                                |
|              | Symbolic dynamics: modified conditional entropy, non-uniform case                                     |
|              | Symbolic dynamics: modified conditional entropy, uniform case                                          |
|              | Symbolic dynamics: forbidden words, non-uniform case                                                  |
|              | Symbolic dynamics: forbidden words, uniform case                                                     |
|              | Symbolic dynamics: Shannon entropy, non-uniform case                                                  |
|              | Symbolic dynamics: Shannon entropy, uniform case                                                     |
|              | Symbolic dynamics: percentage of 0 variations sequences, non-uniform case                             |
|              | Symbolic dynamics: percentage of 0 variations sequences, uniform case                                |
|              | Symbolic dynamics: percentage of 1 variations sequences, non-uniform case                             |
|              | Symbolic dynamics: percentage of 1 variations sequences, uniform case                                |
|              | Symbolic dynamics: percentage of 2 variations sequences, non-uniform case                             |
|              | Symbolic dynamics: percentage of 2 variations sequences, uniform case                                |
| **Geometric** | Dynamic moment of the second order                                                                   |
|              | Dynamical moment of the third order along the principal bisector                                      |
|              | Dynamical moment of the third order along the secondary bisector                                      |
|              | Dynamical moment of the third order along the x-axis                                                 |
|              | Dynamical moment of the third order along the y-axis                                                 |
|              | Finite growth rates                                                                                  |
|              | Grid transformation feature: grid count                                                              |
|              | Poincaré plot SD1                                                                                     |
|              | Poincaré plot SD2                                                                                     |
|              | Poincaré plot cardiac sympathetic index                                                               |
|              | Poincaré plot cardiac vagal index                                                                     |
|              | Recurrence quantification analysis: average diagonal line                                             |
|              | Recurrence quantification analysis: maximum diagonal line                                             |
|              | Recurrence quantification analysis: maximum vertical line                                             |
|              | Recurrence quantification analysis: determinism/recurrences                                           |
|              | Recurrence quantification analysis: percentage of determinism                                         |
|              | Recurrence quantification analysis: percentage of laminarity                                           |
|              | Recurrence quantification analysis: percentage of recurrences                                         |
|              | Recurrence quantification analysis: Shannon entropy of the diagonals                                  |
|              | Recurrence quantification analysis: Shannon entropy of the vertical lines                            |
|              | Recurrence quantification analysis: trapping time                                                     |
| **Energetic** | Low frequency/high frequency ratio                                                                   |
|              | Low frequency (LF) power                                                                              |
|              | High frequency (HF) power                                                                             |
|              | Hjorth parameters: activity                                                                           |
|              | Hjorth parameters: complexity                                                                          |
|              | Hjorth parameters: mobility                                                                            |
|              | Multifractal spectrum cumulant of the first order                                                     |
| Multifractal spectrum cumulant of the second order | Multifractal spectrum cumulant of the third order |
|-------------------|-------------------|
| Multiscale time irreversibility asymmetry index | Plotkin and Swamy energy operator: average energy |
| Teager energy operator: average energy | Very low frequency power |
| Wavelet area under the curve | Informational: Allan factor distance from a Poisson distribution |
| Fano factor distance from a Poisson distribution | Fuzzy entropy |
| Grid transformation feature: AND similarity index | Grid transformation feature: time delay similarity index |
| Grid transformation feature: weighted similarity index | Index of variability distance from a Poisson distribution |
| Kullback-Leibler permutation entropy | Multiscale entropy |
| Multiscale entropy | Predictive feature: error from an autoregressive model |
| Sample entropy | Shannon entropy |
| Shannon entropy | Similarity index of the distributions |
| Invariant: Correlation dimension global exponent | Detrended fluctuation analysis: a1 |
| Detrended fluctuation analysis: a2 | Detrended fluctuation analysis: area under the curve |
| Detrended fluctuation analysis: overall a | Diffusion entropy |
| Embedding scaling exponent | Kolmogorov-Sinai entropy |
| Higuchi scaling exponent | Largest Lyapunov exponent |
| Power Law (based on frequency) slope x2 | Power Law (based on frequency) y-intercept x2 |
| Power Law (based on frequency) x-intercept x2 | Power Law (based on frequency) goodness of fit x2 |
| Power Law (based on histogram) slope | Power Law (based on histogram) y-intercept |
| Power Law (based on histogram) x-intercept | Power Law (based on histogram) goodness of fit |
| Rescaled detrended range analysis | Scale-dependent Lyapunov exponent slope |
| Scale-dependent Lyapunov exponent mean value | Scaled windowed variance |

* LF=[0.04-0.2 Hz]; ** HF=[0.2-2 Hz]; *** VLF=[0.001-0.04 Hz].

# CIMVA= continuous individualized multiorgan variability analysis.
Input fHRV Data

Windowing of Input Data

Waveform Pre-processing and Artifact Detection

R Peak Detection

Artifact Detection and Removal

CIMVA Core

Post-processing

CIMVA Outputs (fHRV measures matrix)

Time domain
- RMSSD,
- probability distributions/frequency histograms

Frequency domain
- high frequency (HF)
- low frequency (LF)
- LF/HF ratio
- total power

Time-frequency domain
- Wavelet area under the curve (AUC)

Scale Invariant (Fractal) Domain
- Power Law
- detrended fluctuation analysis

Entropy domain
- sample entropy
- multiscale entropy
- complexity

Non-linear domain
- Poincare
- Frequency Weighted Energy
Raw data:

- DNA Methylation
- Metabolome
- Microbiome
- ANS

Preprocessing and feature extraction:

- Features

Integrate/fuse features by chosen linear/nonlinear techniques:

- FINAL Features

Can re-design the hypothesis and model, even recollect data:

Training a selected model:

- Learning Model
  - Could be deep learning (Support vector machine, random forest, neural network, boosting, etc.)

Test/validate:

- Research Conclusion

Clinical trial:

FINAL CONCLUSION
Healthy phenotype

\[ z = HR \text{ variability} \]

\[ x = \text{Epigenome} \]

\[ y = \text{Microbiome} \]

Healthy phenotype?

Stress signature?

Healthy phenotype?