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Foundations in human development: Investigating the epigenetic roadmap in gestational and perinatal tissues

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List of abbreviations

BET Betamethasone study

CRH corticotropin-releasing hormone

CVS chorionic villi samples

DNAm DNA methylation

DOHaD Developmental Origins of Health and Disease

eQTL expression quantitative trait loci

eQTM expression quantitative trait methylation

GoDMC Genetics of DNA Methylation Consortium

GTEx Genotype-Tissue expression project

GWAS genome-wide association study

HPA axis hypothalamus-pituitary-adrenal axis

HSD11B2 11beta-hydroxysteroid dehydrogenase type 2

ITU InTraUterine sampling in early pregnancy

meQTL methylation quantitative trait loci

NR3C1 Nuclear Receptor Subfamily 3 Group C Member 1 (glucocorticoid receptor)

PACE Pregnancy And Childhood Epigenetics consortium

PAR predictive adaptive response

PREDO Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction

QTL quantitative trait loci

List of publications

Yusupov, N., **Dieckmann, L.**, Erhart, M., Sauer, S., Rex-Haffner, M., Kopf-Beck, J., Bruckl, T. M., Czamara, D., & Binder, E. B. (2023). Transdiagnostic evaluation of epigenetic age acceleration and burden of psychiatric disorders. *Neuropsychopharmacology*. https://doi.org/10.1038/s41386-023-01579-3

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Contribution to the publications

Contribution to paper I

My first paper (Dieckmann et al., 2021) entitled “Characteristics of epigenetic aging across gestational and perinatal tissues” was published in *Clinical Epigenetics* in 2021. I analyzed and interpreted the data, prepared and edited tables and figures and wrote and edited the manuscript. The co-authors ML-P and JL contributed to data acquisition, pre-processing and interpretation. TK contributed to the collection and pre-processing of ITU cohort data. PD is the author of one of the R packages used for analysis and helped in preparing the code for part of the analyses. CC, HL, SS, PMV, SS-K, JE and EK contributed to data acquisition. KR and EB acquired funding and data, contributed to the design of the work and data interpretation. DC conceptualized the research and contributed to pre-processing, analysis and interpretation of data. All authors were invited to suggest edits for the manuscript and all authors read and approved the final manuscript.

Contribution to paper II

The second paper (Dieckmann et al., 2022) entitled “Reliability of a novel approach for reference-based cell type estimation in human placental DNA methylation studies” was published in *Cellular and Molecular Life Sciences* in 2022. I contributed to the study conception, performed the analyses including preparation of figures and tables, and wrote and edited the manuscript. The authors DC, EB and KR also contributed to the study conception and DC also contributed to data analyses. Funding was acquired by SE, TB, JL, ML-P, KR and EB. Furthermore, CC, ML-P, JL, TK, HL, SS, PMV, SS-K, RCR, AP, WH, JGE, EK, SE, TB and KR were involved in the collection and preparation of material. All authors read and commented on the manuscript draft and approved the final version.

1. Introductory summary

1.1 The impact of early life on health

1.1.1 Developmental programming

Developmental programming suggests that the prenatal environment can influence fetal development with long-lasting effects on future development and health.

Originally, David Barker proposed the *fetal origins hypothesis* based on his observation that undernutrition during pregnancy can affect fetal growth and predispose the child to cardiac and metabolic disorders in adulthood (Barker, 1995; Barker et al., 2002). Godfrey and Barker (2001) outlined these proposed associations based on evidence from different studies: Undernutrition in the womb may cause inadequate fetal and placental growth, leading to low birth weight and, in an attempt to catch-up after birth, to accelerated growth during childhood. This was in turn associated with elevated blood pressure and a higher likelihood of developing hypertension in adult life, thus increasing the risk for coronary heart disease and related disorders. Later, the *fetal origins hypothesis* was expanded and formalized in the *Developmental Origins of Health and Disease (DOHaD) hypothesis*. The DOHaD hypothesis states that the prenatal environment may permanently program the structure and physiology of the offspring during critical periods *in utero*, thereby influencing later health outcomes (Barker, 2007; Gluckman & Hanson, 2004). To adapt to adverse prenatal conditions, fetal metabolism and endocrinology may change, delaying growth but ensuring immediate survival (Barker, 1998; Kwon & Kim, 2017). This ability of an organism to respond to environmental cues is known as developmental plasticity, and is evolutionarily adaptive (Barouki et al., 2012). Thus, it is important to not only consider the detrimental but also the adaptive consequences of fetal programming when studying this phenomenon. Accordingly, the *predictive adaptive response (PAR) hypothesis* suggests that cues in early life favor the development of a well-adapted phenotype if the conditions later in life match the early environmental conditions (Gluckman et al., 2005). On the other hand, if the predicted and actual environment differ, the mismatch between prepared phenotype and environmental conditions can increase the risk for adverse health outcomes (Bateson et al., 2014).

Together, these concepts highlight the importance of the earliest developmental phase in understanding later health trajectories and individual responses to health challenges. The DOHaD theory has been supported by evidence from both animal and human studies (McMullen & Mostyn, 2009) and can guide future research (Hagemann et al., 2021; Wadhwa et al., 2009). In fact, it has been expanded to many different research fields, such as molecular and developmental biology, human genetics and epidemiology (Suzuki, 2018). Finally, developmental programming has implications for designing early prevention and intervention programs and is highly relevant for public healthcare (Heindel et al., 2015; Jacob & Hanson, 2020). Global health initiatives, for instance, have noted and heeded evidence supporting developmental programming (Black et al., 2017; Clark et al., 2020; Kuruvilla et al., 2016). Investments during the pregnancy period have been proposed to be important, both from a social and economic perspective (Doyle et al., 2009; Every Woman Every Child, 2015; Penkler et al., 2019).

1.1.2 Pregnancy as a sensitive period

Over the past years, the range of both prenatal influences and later-life outcomes investigated in the field of developmental programming has increased (see Abdul-Hussein et al., 2021; Ramirez et al., 2022). In addition to the aforementioned association between fetal undernutrition and cardiac and metabolic diseases, fetal undernutrition has been further linked to cognitive performance and related outcomes later in life (Fall, 2013; Hoffman et al., 2017; Roseboom, 2019; Victora et al., 2008). Furthermore, several maternal characteristics have been related to child development. For example, maternal pre-pregnancy obesity has been linked to attention-deficit/hyperactivity disorder in the offspring (Li et al., 2020), maternal hypertensive disorders and preeclampsia during pregnancy have been associated with child neurocognitive outcomes (Figueiró-Filho et al., 2017), and maternal prenatal stress – or more generally maternal well-being – has been found to be an important determinant for offspring neurodevelopment and mental health later in life (Bale et al., 2010; O'Donnell & Meaney, 2017; Van den Bergh et al., 2020). Mental disorders linked to prenatal stress include autism spectrum disorder, attention-deficit hyperactivity disorder, depression, anxiety, and schizophrenia (Lautarescu et al., 2020; Manzari et al., 2019; O'Donnell et al., 2009; Tuovinen et al., 2021). Furthermore, external environmental influences during pregnancy can have long-lasting impacts on the child (Almeida et al., 2019). For instance, prenatal chemical exposure has been associated with poor neurodevelopmental outcomes (Bellinger, 2013; Tohyama, 2019), air pollution has been reported to adversely affect cardiovascular, metabolic, respiratory and neurodevelopmental outcomes (Gheissari et al., 2022), and maternal infection and immune activation have been related to a higher risk for autism and schizophrenia-related symptoms in the child (Haddad et al., 2020; Jiang et al., 2016). On the other hand, a healthy maternal lifestyle and environment can promote positive outcomes. For example, exercise during pregnancy has been found to be beneficial for fetal and postnatal health (Moyer et al., 2016). However, early resiliency factors are still underexplored (Abdul-Hussein et al., 2021).

In sum, a range of prenatal exposures reportedly affect the future health status and development of the offspring (Özturk & Turker, 2021). Pregnancy is an important and especially sensitive period in determining postnatal outcomes. The pace of fetal development exceeds that of any other period during the life span, and fetal brain maturation in particular is rapid (Davis & Narayan, 2020). During the late fetal phase, for instance, approximately 40,000 new synapses are formed per second, and the fetal period is essential for neuron production, migration, connection and differentiation (Monk et al., 2019). Because these foundations of brain development are laid prenatally, it is plausible that pregnancy constitutes a susceptible period that can have long-lasting effects on health (Cruceanu et al., 2017). Like the brain, other fetal organs are susceptible to change during gestation (Sly et al., 2021). For example, prenatal air pollution exposure has been linked to poor lung function and growth, which can increase the risk of respiratory symptoms (Hsu et al., 2023; Korten et al., 2017); and prenatal malnutrition has been reported to impair endocrine pancreas development, which can increase the risk of diabetes (Moullé & Parnet, 2019; Remacle et al., 2007). Changes in the structure and function of cells, tissues and fetal organs could explain the observed long-lasting consequences of prenatal conditions for health outcomes (Barker et al., 1993; Godfrey, 2002). Still, questions about the mechanisms underlying these changes remain. How can we explain developmental programming on a molecular level?

1.1.3 Mechanisms of developmental programming

1.1.3.1 Importance of epigenetics

The underlying mechanisms of developmental programming are not yet completely understood, but researchers suspect that epigenetic processes play an important role (Arima & Fukuoka, 2020; Stevenson et al., 2020). Epigenetics is the study of changes in gene activity that are mitotically heritable and not caused by changes in the DNA sequence itself (Jaenisch & Bird, 2003). The three main epigenetic mechanisms are DNA methylation (DNAm), histone modifications and noncoding RNAs (Gibney & Nolan, 2010; Goyal et al., 2019).

Epigenetic modifications establish a connection between environmental cues and phenotypic outcomes by changing gene expression patterns in a cell- and tissue-specific manner (Feil & Fraga, 2012; Hoffman et al., 2017). The link between environment and epigenome involves receptor signaling, energy metabolism and signal mechanotransduction from extracellular matrix to chromatin (Safi-Stibler & Gabory, 2020). Epigenetic mechanisms act on both the fetal and maternal sides. However, the epigenome is particularly vulnerable during prenatal development, when extensive programming and reprogramming of epigenetic modifications occur (Kundakovic & Jaric, 2017).

Environmental influences can trigger hypothalamus-pituitary-adrenal (HPA) axis activation, immune activation, microbiome dysregulation (Monk et al., 2019) or metabolic changes (Parrettini et al., 2020) in the mother. Processes involving the HPA axis and stress response are among the most researched and understood. The HPA axis is one of the main systems implicated in the stress response (see Arnett et al., 2016; Packard et al., 2016; Russell & Lightman, 2019). Thus, consequences of HPA axis dysfunction are relevant both in the mother and fetus. Alterations in HPA axis functioning have been related to inappropriate stress responses and neuropsychiatric disorders (Kinlein et al., 2015; McEwen, 2004; Sheng et al., 2020; Zorn et al., 2017).

Evidence from animal and human studies implicates maternal prenatal stress as a source of epigenetic modifications, which potentially affect fetal brain development and may program the risk for emotional dysregulation and mental disorders over a lifetime (DeSocio, 2018). These long-term effects of prenatal stress are at least partially mediated by glucocorticoids, HPA axis functioning and epigenetic regulation of involved genes (Krontira et al., 2020; Matthews & McGowan, 2019; Provencal & Binder, 2015). For example, one of the most commonly studied candidate genes is the glucocorticoid receptor gene *NR3C1*. DNAm levels of *NR3C1* in the offspring have been associated with prenatal stress exposure (Palma-Gudiel, Córdova-Palomera, Eixarch, et al., 2015; Sosnowski et al., 2018; Turecki & Meaney, 2016). In turn, epigenetic modification of this gene may impair HPA axis functioning, thereby increasing the risk for psychiatric disease (Palma-Gudiel, Córdova-Palomera, Leza, et al., 2015).

The transmission of maternal exposures and experiences to the child via epigenetic pathways is mediated by the placenta, which will be the focus of the next section.

1.1.3.2 The crucial role of the placenta

The special role of the placenta was postulated by Barker from the very beginning of his investigations of fetal programming (Barker, 1998). As a regulator of fetal nutrient supply and growth, the placenta is critical for fetal development and programming. The placenta is a metabolic and endocrine organ and mediates solution transfer actively (Burton et al., 2016; Parrettini et al., 2020). Epigenetic regulation plays an important role in placental development and functions

(Koukoura et al., 2012; Nelissen et al., 2011). These functions include maintaining an immunological barrier between fetus and mother, mediating the transfer of respiratory gases, water, ions and nutrients, and producing and secreting hormones, cytokines and signaling molecules (Jansson & Powell, 2007). Environmental modulation during pregnancy involves morphological and functional adaptions of the placenta with downstream effects on the offspring (Sferruzzi-Perri & Camm, 2016). The fetal or perinatal responses that might affect the development of fetal organs include changes in metabolism, hormone production and tissue sensitivity to hormones (Barker et al., 1993; Gluckman et al., 2008).

Epigenetic signatures in human placental tissue have been associated with a range of maternal characteristics and environmental exposures, including maternal psychopathology, endocrine and metabolic status, nutrition, stress and smoking during pregnancy, and exposure to environmental pollutants (Cleal et al., 2022; Palma-Gudiel et al., 2018). Furthermore, placental DNAm levels appear to change during gestation, possibly in response to changing cellular composition and cumulative environmental influences (Novakovic et al., 2011). While placental changes can represent adaptive mechanisms to protect the developing fetus from detrimental exposures, they have the potential to program the child in such a way that will later increase disease risk (Cleal et al., 2022; Nugent & Bale, 2015). Moreover, the placenta may only be able to protect the fetus from adverse exposures to a certain extent.

The relationship between prenatal exposure and placental regulation can be demonstrated using prenatal stress as an example. The human placenta expresses corticotropin-releasing hormone (CRH), a major stress hormone (King et al., 2001). During pregnancy, maternal CRH is largely derived from placenta secretion and triggers secretion of maternal cortisol, which further activates the release of CRH from the placenta in a positive feedback loop (Alcantara-Alonso et al., 2017; Robinson et al., 1988). Increasing stress hormone levels throughout pregnancy constitute a normal process important for fetal maturation (Chatuphonprasert et al., 2018; Mastorakos & Ilias, 2003; Pofi & Tomlinson, 2020). Yet, elevated stress hormone levels during pregnancy have been associated with fetal brain structure alterations, thereby potentially programming the fetal nervous system (Kassotaki et al., 2021; Sandman et al., 2011). The exposure of the fetus to glucocorticoids is controlled by placental expression of the enzyme 11beta-hydroxysteroid dehydrogenase type 2 (HSD11B2), which converts cortisol to inactive cortisone, thus buffering maternal glucocorticoid access to the child (Benediktsson et al., 1997; Sun et al., 1999; Togher et al., 2014; Wyrwoll et al., 2011). However, chronic maternal prenatal stress has been associated via placental DNAm with a decrease in HSD11B2 gene expression, thus reducing this protective effect (Jahnke et al., 2021; Jensen Pena et al., 2012). Consequently, stress during pregnancy could disturb maternal stress response systems and change epigenetic regulatory processes. This may influence placental glucocorticoid metabolism, which in turn has an impact on offspring neurodevelopment.

Notably, the importance of the placenta for fetal brain development – and implications for neurobehavioral disorders – has been increasingly recognized during recent years and was conceptualized in the term ‘placenta-brain-axis’ (Rosenfeld, 2021). Due to increasing evidence of the placenta as an active brain architect, the placenta was even referred to as a ‘third brain’ by Lester and Marsit (2018). In sum, the placenta is involved in the formation of the developing fetus and has an outstanding role in the orchestration of fetal-maternal interactions (Lapehn & Paquette, 2022; Sferruzzi-Perri & Camm, 2016; Shallie & Naicker, 2019).

1.1.4 Factors influencing the relationship among prenatal exposure, epigenetics and child development

When considering potential mechanisms underlying developmental programming, researchers should take into account a variety of factors that can modify and refine some of the observed associations between prenatal exposures, (placental) epigenetics and child developmental outcomes. The most critical factors (apart from more technical considerations) are described in this section.

First, fetal characteristics such as sex and genetic predisposition can influence the child's susceptibility to different exposures. Sex-dependent differences were observed in placental function, implicating sex-specific placental responses (Rosenfeld, 2015). For instance, the effect of prenatal stress can vary by the child's sex (Jahnke et al., 2021; Stoye et al., 2020). Furthermore, there is a complex interplay between genetic, epigenetic and gene expression variability (Bollati & Baccarelli, 2010; Capp, 2021). For example, it has been shown that interactions between genetic and environmental factors (GxE) can explain more DNAm variability in perinatal tissues than genotype or environmental factors alone (Chatterjee et al., 2021; Czamara et al., 2019; Teh et al., 2014).

Second, the effect of an exposure – for instance, prenatal stress – can be influenced by the timing of its occurrence (see Bronson & Bale, 2016; Lautarescu et al., 2020; Van den Bergh et al., 2020). In general, there are different susceptible periods for different influences, and the downstream effects of an exposure often depend on the timepoint of its occurrence during pregnancy (Davis & Narayan, 2020; Langley-Evans, 2004).

Third, beyond pregnancy, the delivery process itself should be considered as a contributor to epigenetics and developmental outcomes in the child. For instance, cesarean section has been associated with an increased risk of various diseases in later life, and one explanatory hypothesis suggests that the mode of delivery could affect the epigenetic state of stem cells in newborns (Almgren et al., 2014; Linnér & Almgren, 2020). Furthermore, the delivery mode might influence early gut microbiota composition, with implications for immune disorders in the child (Kristensen & Henriksen, 2016; Lee, 2019).

Overall, the earliest period in life can prime the developing child to respond differently to environmental exposures over the life course. Thus, studying the early life period can offer insights into the underlying causes of different susceptibilities to future experiences. Prenatal influences include a range of environmental exposures and maternal characteristics, which should be considered together with characteristics of the child, pregnancy and birth processes. Epigenetic mechanisms, especially, but not only, in the placenta, have the potential to provide insights into the pathways underlying this transmission of prenatal environment to child phenotypes. This is summarized in Figure 1.

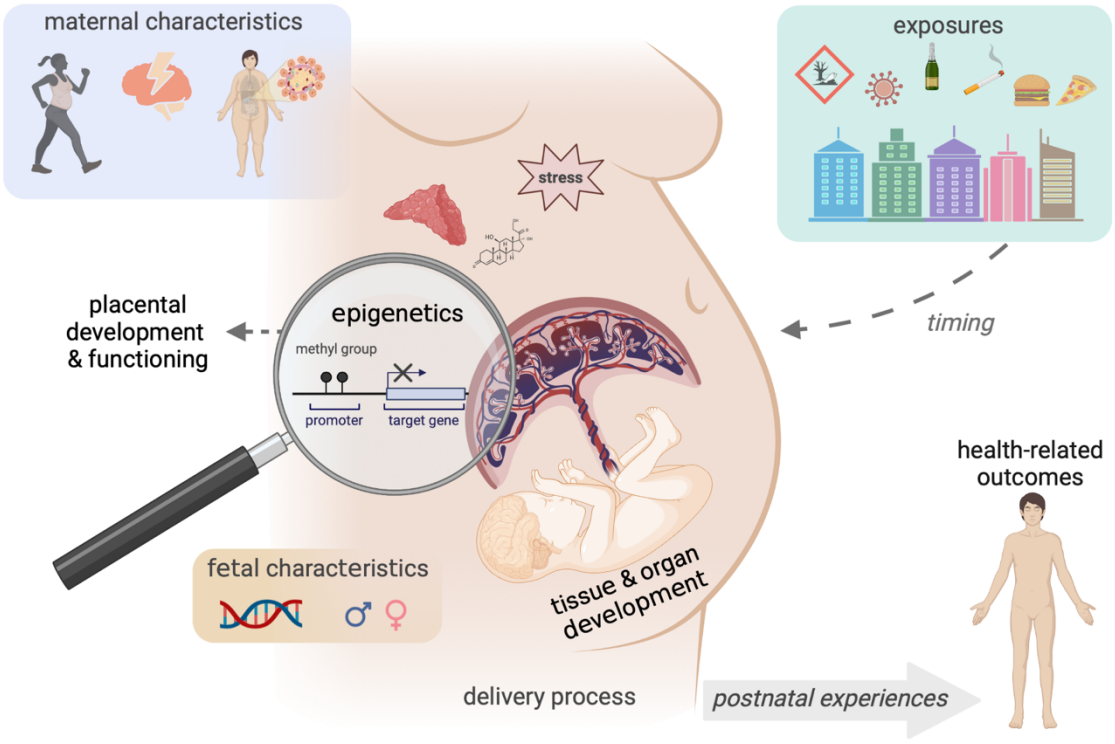

Figure 1. Illustration of developmental programming: Several factors, such as maternal characteristics and environmental exposures, have been associated with fetal developmental outcomes. Epigenetic mechanisms (e.g., DNA methylation) suggest a pathway of how these factors can translate to placental and fetal biology. The placenta is crucial for fetal development, and changes in placental development and functioning may affect fetal tissue and organ development. This could, in turn, program the offspring's physiology, with long-term consequences for health. Figure created with BioRender.com.

1.2 Approaches to study prenatal human development

1.2.1 Model systems and cohort studies

Both research in animal models and humans contributed to understanding the impact of early life on health. Animal models can be used to study specific mechanisms, and can potentially show causality, in a wide range of tissues including the brain. However, translating these findings to humans is challenging (Frangogiannis, 2022; McGonigle & Ruggeri, 2014). Another promising modeling approach are *in vitro* cell cultures, which include three-dimensional organoids (de Souza, 2018; Richardson et al., 2020). These were successfully used to model certain brain regions (Bassil et al., 2023; Luo et al., 2016) and have also been applied to model placental tissue (Gundacker & Ellinger, 2020; Turco & Moffett, 2019). Nevertheless, human studies remain crucial for gaining insight into the full complexity of human development.

Epidemiological cohort studies have been one of the main methods of studying developmental programming in humans, and large-scale prospective birth cohort studies will continue to be important (Sata, 2019; Suzuki, 2018). Such studies are well suited to identify exposures and risk factors before disease onset and to detect gene-environment interactions related to disease (Manolio et al., 2006). Although cohort studies entail challenges too, they provide more unbiased research in a natural setting and are very powerful when they involve different environmental factors and phenotypes including molecular data (Wijmenga & Zhernakova, 2018).

1.2.2 Gestational and perinatal tissue samples

Human research primarily relies on accessible tissues due to concerns about ethics and invasiveness. The placenta is of particular interest when studying pathways of developmental programming. Other peripheral tissues commonly used include buccal cells and cord blood (Sosnowski et al., 2018). Gestational and perinatal tissues, sampled directly after birth or even during pregnancy, help to gain insights into the prenatal development, as later sampling points most likely also reflect postnatal influences.

The biological relevance of a tissue for the studied phenomenon is important to consider. As discussed previously, the placenta is highly relevant in developmental programming. Thus, placental biomarkers have the potential to identify prenatal exposures and their relation to child development and disease risk (Cleal et al., 2022; Manokhina et al., 2017). Generally, gestational and perinatal tissues are target tissues for studies of prenatal development, and may even deliver information about processes in other, less accessible, fetal tissues. Often it is unknown whether a biomarker is simply an epiphenomenon or actually a component of a mechanistic pathway – consequently, surrogate biomarkers are not necessarily inferior (Hanson & Gluckman, 2014). Nevertheless, it is important to keep in mind that findings in one tissue cannot simply be extrapolated to other tissues.

Although the placenta is easily accessible after birth, it is among the most poorly researched organs (Benirschke, 2004; Bhattacharya et al., 2022; Shallie & Naicker, 2019). However, awareness of the lack of knowledge about placenta biology, and research efforts, have increased. For example, the 'Human Placenta Project' was initiated to better understand placental functioning during gestation (Guttmacher et al., 2014). Furthermore, placental DNAm data is now available in a subset of cohorts included in the PACE (Pregnancy And Childhood Epigenetics) consortium (Felix et al., 2018), providing a resource for further investigations. Still, placental tissue is absent from some large-scale cohort studies and important established consortia, such as the GTEx (Genotype-Tissue expression) consortium (GTEx Consortium, 2019) and the Genetics of DNA Methylation Consortium (GoDMC; Min et al., 2021). It is noteworthy that placental tissue sampling is challenging due to regional differences that need to be considered and can lead to heterogenous tissue samples (Burton et al., 2014). Actually, the placenta is one of the most structurally diverse organs within all mammals (Rosenfeld, 2021).

Importantly, placental tissue should not only be studied post-delivery but also during gestation (Guttmacher & Spong, 2015). During pregnancy, the placenta is rapidly developing (Burton et al., 2009; Cindrova-Davies & Sferruzzi-Perri, 2022). Formed from the zygote at the start of pregnancy, the placenta has the same genetic composition as the fetus (Burton & Fowden, 2015; Herrick & Bordoni, 2022). The zygote becomes a blastocyst, consisting of an inner cell mass and a trophectoderm (Kojima et al., 2022). The trophoblast cell layer of the blastocyst proliferates into cytotrophoblast and syncytiotrophoblast cells (Carlson, 2014). Both cytotrophoblast and syncytiotrophoblast are part of the chorion (Herrick & Bordoni, 2022). From the chorion, vascular projections of fetal tissue form chorionic villi, which are the basic structural unit of the placenta and project into maternal blood (Griffiths & Campbell, 2015; Ventura Ferreira et al., 2018). Chorionic villi consist of an outer layer of syncytiotrophoblast (in contact with maternal blood), a layer of cytotrophoblasts, connective tissue and the fetal vascular endothelium (Herrick & Bordoni, 2022; Lewis et al., 2017). They not only provide a barrier between fetal and maternal blood systems but are also essential for the exchange of gas, nutrients and waste between fetus and mother (Caruso et al., 2012; Maltepe & Fisher, 2015; Mori et al., 2007). In order to further facilitate fetomaternal exchange, these villi undergo dynamic morphological changes throughout gestation (Knöfler et

al., 2019; Mori et al., 2007). To study the human placenta in an earlier pregnancy stage, extremely valuable though rare resources are first trimester chorionic villi samples (CVS), which are sampled for early prenatal genetic testing if medically indicated (Hannibal et al., 2018; Vink & Quinn, 2018).

1.2.3 Epigenetic patterns

1.2.3.1 DNA methylation

In principle, all of the different epigenetic processes, including DNAm, histone modifications and noncoding RNAs, are of interest in investigating gene regulation. However, the most studied and best understood epigenetic modification in humans is DNAm (Haugen et al., 2015; Hoffman et al., 2017). Furthermore, the studies of this thesis are centered around DNAm. Hence, this section focuses on DNAm and corresponding study approaches.

DNAm is an epigenetic mark, referring to the transfer of a methyl group onto cytosine residues and mainly occurring in the context of CpG dinucleotides (Bird, 1986; Dor & Cedar, 2018; Moore et al., 2013). The necessary writing and removal of methyl groups, and the translation into functional information, is performed by a machinery of proteins in the cells (Dor & Cedar, 2018). DNAm can regulate gene expression by modulating the binding of transcription factors to regulatory DNA elements (e.g., promoters, enhancers) and determining patterns of histone modifications, thereby controlling transcriptional states (Héberlé & Bardet, 2019; Rose & Klose, 2014; Yin et al., 2017). Typically, DNAm has been associated with repressed gene transcription, but growing evidence indicates that the manner in which DNAm influences transcriptional activity is more complex and appears to be context (e.g., DNA properties, stimuli) dependent (de Mendoza et al., 2022; Dhar et al., 2021; Smith et al., 2020).

Differentiated cells develop a unique and relatively stable DNAm pattern that is important for the regulation of tissue-specific gene expression (Khavari et al., 2010; Moore et al., 2013). Thus, an essential function of DNAm is to maintain cell-specific gene expression patterns established during embryonic development (Lande-Diner et al., 2007; Razin & Szyf, 1984; Santos & Dean, 2004). This is crucial for the development and function of an organism (Robertson, 2005). Still, DNAm is dynamic to some extent and responsive to environmental influences (Jaenisch & Bird, 2003; Leenen et al., 2016; Martin & Fry, 2018; Meaney & Szyf, 2005). Different environmental cues, such chemical compounds (e.g., pollutants), lifestyle factors (e.g., nutrition, smoking, alcohol consumption) and stress have been associated with DNA methylation changes, which can affect epigenetic gene regulation and consequently phenotypes (Feil & Fraga, 2012). In sum, DNAm marks reflect an interaction among environment, epigenome and genome (Dhar et al., 2021).

Common methods to measure DNAm can be categorized into three main approaches: global methylation levels, targeted gene sequencing, and genome-wide methylation screening (Colwell et al., 2023). In epidemiological cohort studies, epigenome-wide approaches using popular and affordable array-based platforms are common (Campagna et al., 2021; Colwell et al., 2023; Shu et al., 2020). DNAm arrays used in most of these studies cover only a fraction of the human methylome, and some potentially missed signals might be detected when advanced technologies become affordable in the future (Shu et al., 2020; Stevenson et al., 2020).

For any DNAm analysis in bulk tissue, it is extremely important to take into account that differences in DNAm levels between samples can occur due to variation in the cell type composition

(Jaffe & Irizarry, 2014; Ohgane et al., 2008). Cell type deconvolution algorithms can help to address this issue in samples derived from heterogenous mixtures of cells (Titus et al., 2017). Above that, single-cell studies are needed to further improve the understanding of cell type-specific effects (Karemaker & Vermeulen, 2018).

Overall, methods and approaches to use DNAm to better understand the pathways linking environmental exposures and changes in phenotypes continue to develop. Several challenges – involving tissue specificity and cell type adjustment, issues of power and comparability of findings, genetic influences, stochastic epigenetic variation, functional impact and causality – have to be addressed (Felix & Cecil, 2019; Poulsen et al., 2007). Despite these challenges, the field of population epigenetics is evolving, and is promising, especially in investigations of how earliest life influences map to measurable molecular changes on the tissue level.

1.2.3.2 Epigenetic clocks as DNA methylation-based biomarkers

An important research aim in the field of population epigenetics is the development of DNAm-based biomarkers. Biomarkers can be defined as objective, quantifiable characteristics of biological processes (Strimbu & Tavel, 2010). They have been developed using DNAm data for different fields of application (Nwanaji-Enwerem & Colicino, 2020; Wagner, 2022). The focus of this thesis will be on a biomarker for aging, which is relevant for the concept of developmental programming and gained popularity in the recent decade: the epigenetic clock.

Epigenetic clocks are designed to estimate biological age from DNAm at selected CpG sites (Horvath, 2013; Horvath & Raj, 2018). They evolved from a combination of factors: the observation that DNAm changes with age, the better availability of large DNAm data sets, and the need for biomarkers for aging (Horvath & Raj, 2018). The first two epigenetic clocks were developed independently by Horvath (2013) and Hannum et al. (2013), and trained to predict chronological age. The difference between chronological age and estimated DNAm age is referred to as age acceleration or deceleration (Horvath, 2013). It can be interpreted as an epigenetic measure of a relatively faster or slower biological aging process. Until now, several epigenetic clocks have been built using different methods, tissues, outcome measures and purposes (see Bergsma & Rogaeva, 2020; Declerck & Vanden Berghe, 2018; Oblak et al., 2021; Salameh et al., 2020; Topart et al., 2020). These clocks contain different proportions of chronological and biological information, and their specific focus might become more precise in the future (Bell et al., 2019; Bernabeu et al., 2023; Field et al., 2018).

In adults, epigenetic aging has been related to a range of lifestyle factors and health-related phenotypes, including alcohol and cigarette consumption, diet, stress, sex, physical activity, neurological disorders, cancer and mortality (Dhingra et al., 2018; Galow & Peleg, 2022; Oblak et al., 2021; Topart et al., 2020). Hence, by capturing molecular processes related to biological aging, epigenetic clocks serve as powerful tools to study aging, development and health across the lifespan (Godfrey et al., 2015; Ryan, 2020).

However, age-related DNAm dynamics appear to be different in childhood as compared to later life, and epigenetic clocks for pediatric populations are required (Alisch et al., 2012; Wang & Zhou, 2021). Childhood-specific epigenetic clocks were designed from blood (Wu et al., 2019) and buccal cells (McEwen et al., 2019). Epigenetic clocks to estimate gestational age in newborns were developed for cord blood (Bohlin et al., 2016; Haftorn et al., 2021; Knight et al., 2016), and placental tissue (Lee et al., 2019; Mayne et al., 2017). Nevertheless, both the clocks and studies

applying them for the prenatal and early postnatal period have been underrepresented. For example, no investigations have examined how gestational epigenetic age corresponds between different tissues.

Yet epigenetic clocks are promising in evaluations of how environmental and contextual factors (such as prenatal stress or pregnancy conditions) may relate to child development on a biological level. For example, Knight et al. (2016) proposed that epigenetic gestational age can provide information about the developmental state of the newborn. That is, accelerated epigenetic age at birth might indicate higher developmental maturity. In more general terms, gestational epigenetic age acceleration/deceleration could reflect epigenetic programming by early exposures, potentially affecting neonatal outcomes (Wang & Zhou, 2021). Thus, while initially designed as molecular biomarker for chronological age, epigenetic clocks have the potential to function as a useful biomarker to evaluate the impact of early exposures and predict developmental outcomes from early life onwards (Wang & Zhou, 2021).

1.3 Aims and results of this thesis

Previous sections have emphasized the impact of the earliest phase of human development on future health, discussed epigenetic processes as essential mechanisms involved in developmental programming, and outlined the crucial role of the placenta for prenatal fetal development. At the same time, the need for further insights into the human placenta and its complexity has been addressed. Moreover, it has been established that DNAm itself is tissue specific (Varley et al., 2013), which is essential to consider in any study using DNAm data. As one important tool for studying human development and health across the lifespan, epigenetic clocks designed as biomarkers from DNAm data have been introduced.

The first aim of this thesis is to investigate how a variety of prenatal and perinatal influences associate with gestational epigenetic aging in different gestational and perinatal tissues. The second aim is to advance the understanding of human placental tissue samples and how cell type proportions can be considered and estimated from DNAm in these samples. Figure 2 depicts how the studies are embedded in the research field. The following paragraph outlines their contribution to filling current research gaps in more detail.

The first publication (Dieckmann et al., 2021) provides insights into epigenetic aging patterns in both first trimester CVS, term placental tissue, and cord blood. Despite the promising role of epigenetic clocks in investigating aging and development, there is a lack of knowledge about the characteristics of gestational epigenetic aging in newborns, especially regarding different tissue samples and the relative contribution of different potential influences that may associate with gestational epigenetic age. We used two Finnish cohort studies, with a total sample size of over 1,500 samples, to explore which variables were most strongly associated with gestational epigenetic aging. Variables related to birth and pregnancy, as well as child and maternal characteristics were considered. To predict epigenetic age acceleration/deceleration in the respective tissue with these variables, we applied elastic net regression with bootstrapping. We found that relatively higher or lower epigenetic age was not generally related to either more favorable or unfavorable circumstances across the investigated tissues. Furthermore, the variables associated with the relative epigenetic age differed between the tissues. Thus, an important conclusion from our findings is that the estimated gestational epigenetic age should be interpreted as an attribute of the specific tissue and should not simply be generalized to other newborn tissues. Even so, knowledge about the factors related to biological aging patterns of a tissue is important and can

offer even more specific insights into the early influences of human development (i.e., which influences may be particularly strong in this specific tissue). For future studies it would be interesting to further investigate potential pathways from prenatal environment to epigenetic aging and postnatal outcomes of specific variables identified to be important (such as child sex, birth length, maternal smoking or mental disorders, parity and delivery mode). In such studies, results should be interpreted in relation to the specific clock and tissue used.

As discussed previously, cell type proportions are extremely important for DNAm studies in bulk tissues. Furthermore, advancing our understanding of placental epigenetic regulation would be very valuable in the context of developmental programming, but it must be noted that the placenta is a highly complex organ. In order to better understand the molecular profile of placental tissue, we need to consider the related challenges. For instance, the placenta consists of a heterogeneous mix of cell populations within each sampling site (Bianco-Miotto et al., 2016; Lapehn & Paquette, 2022); and the differences in the methylome of each cell type must be accounted for to prevent biased study results (Jaffe & Irizarry, 2014). Although methods have been developed to estimate cell type proportions from DNAm data, such approaches have not been validated in the placenta (Wilson & Robinson, 2018).

The second publication (Dieckmann et al., 2022) aimed to evaluate available cell type deconvolution methods for placental DNAm data and provide insights into estimated cell type composition in placenta samples from different sources. We examined the performance of a newly available reference-based cell type estimation approach (Yuan et al., 2021) together with an established reference-free cell type estimation approach (Houseman et al., 2016) in first trimester (CVS) and birth placenta samples, using three independent studies (InTraUterine sampling in early pregnancy (ITU), Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO), Betamethasone (BET) study), comprising over 1,000 samples. We found that both reference-free and reference-based estimated cell type proportions contributed to the prediction of DNAm levels. However, reference-based cell type estimation outperformed reference-free estimation for the majority of data sets and offers better interpretability by providing further insights into possible histological differences between the placenta samples. In sum, our investigation contributes to a better understanding of cell type compositions in human placenta samples that are reflected in DNAm data. Furthermore, this study provides a resource for future study design and interpretation of results involving human placental DNAm data.

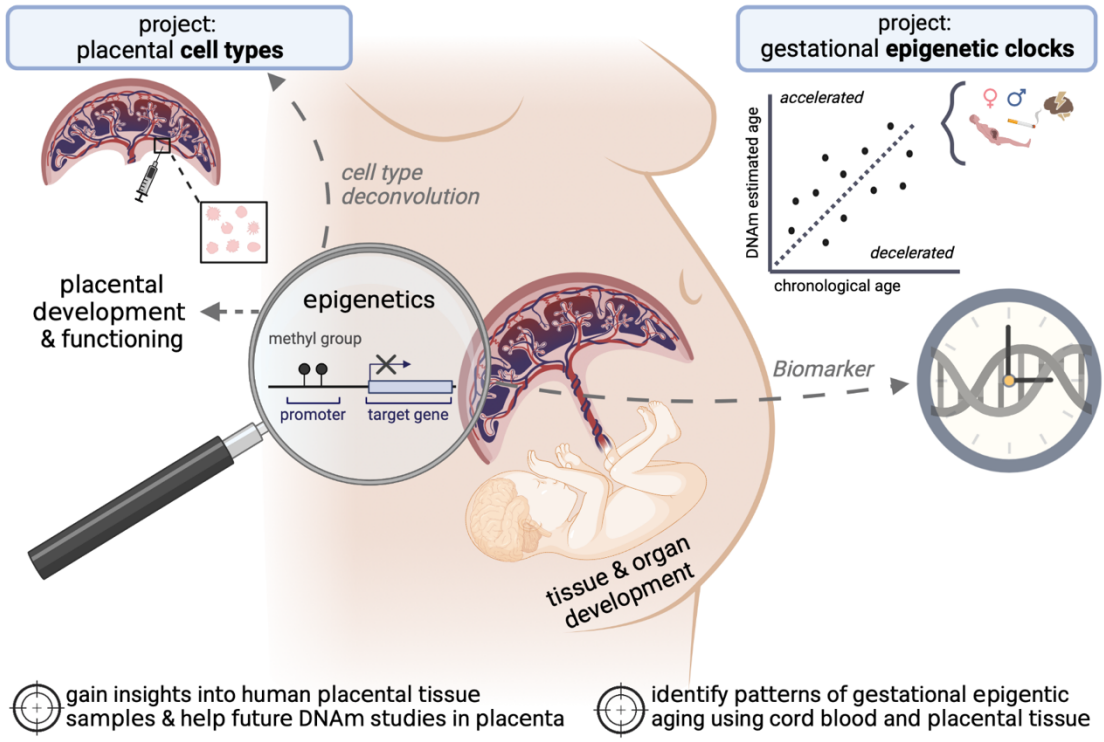

Figure 2. Illustration of the embedding of the projects included in this thesis into the research field. Figure created with BioRender.com.

1.4 Limitations and outlook

Some of the limitations and challenges in the research field have already been mentioned. However, they should be addressed here with a greater focus on the studies included in this thesis and combined with an outlook on future investigations.

As in most human epigenetic studies, the observations are correlative and generally findings should be replicated. Replication can be challenging due to different cohort characteristics and DNAm arrays, the complexity of placental sampling and other aspects of the study design. Nevertheless, future studies must further evaluate both the robustness and generalizability of findings. The increasing interest in placental tissue might offer new opportunities for such analyses as larger study samples with placental tissue become available. Additionally, future studies could further benefit from applying more standardized and optimized placental sampling techniques (Burton et al., 2014).

The interpretation of epigenetic clock analyses largely depends on the design of the underlying epigenetic clock. Epigenetic clocks contain both biological variation (signal) and technical variation (noise), which can be difficult to disentangle. In the future, novel computational solutions and improved designs may further advance epigenetic clocks (Bernabeu et al., 2023; Galow & Peleg, 2022; Higgins-Chen et al., 2022). Apart from that, researchers are only beginning to grasp the mechanisms behind epigenetic aging (Li et al., 2022; Wang & Zhou, 2021). To understand the long-term impact of epigenetic age associations found in tissue samples assessed at birth, longitudinal follow-up investigations are needed. Although it should be mentioned that the postnatal environment needs to be further taken into account in those studies.

Sex and genetic variation are among the most critical factors that are known to influence DNAm (see Govender et al., 2022; Villicana & Bell, 2021). Particularly in studies involving placental tissue, sex-specific differences were observed and should be further explored in future studies (Andrews et al., 2022). Regarding genetic variation, methylation quantitative trait loci (meQTL) provide a promising tool to investigate the links between genetic polymorphisms and DNAm differences (Min et al., 2021). Moreover, integrating different levels of omics data could provide a more complete picture of the molecular profile in a tissue (Kreitmaier et al., 2023). To this end, we are currently investigating placental regulation with different quantitative trait loci (QTL) in the ITU cohort. More specifically, we integrate genetic, epigenetic and transcriptomic data using expression quantitative trait loci (eQTLs), expression quantitative trait methylation (eQTM) and meQTLs in both first trimester (CVS) and birth placental tissue. In general, we identified more QTL associations in birth placenta compared to CVS, while the direction of effects was preponderantly congruent in both tissues. This implies that there are early established genetic regulatory influences that remain stable, while there is an overall increase in the number of regulatory relations among genome, methylome and transcriptome throughout gestation. Furthermore, we will explore how the genetic polymorphisms linked to the quantitative molecular traits overlap with genome-wide association studies (GWAS). This will not only help to understand placental genetic regulation during different pregnancy stages, but also constitutes an important step towards understanding the association of placental regulation with health and disease.

1.5 Conclusion

The studies included in this thesis provide critical insights into the epigenomic profile of gestational and perinatal tissues. They contribute to the investigation of biological pathways involved in developmental programming and approaches to design future studies. Various factors can influence early development, and the impact of several of these factors on epigenetic aging of cord blood and placental tissue was explored. Importantly, not only term placenta but also first trimester placental biopsies were examined. Furthermore, estimated cell type proportions from DNAm were characterized in different placental tissue samples. This is highly relevant for any epigenetic study involving DNAm in bulk placental tissue. The placenta is possibly the most central tissue to understand the epigenetic mechanisms involved in developmental programming (Lapehn & Paquette, 2022; Marsit, 2016), and is proposed to show an environmental ‘memory’ of the circumstances during pregnancy (Novakovic & Saffery, 2012). Thus, studies investigating the relationship among the placenta, epigenetics, and developmental programming will likely increase in the coming years. A more complete understanding of the molecular landscape of placental tissue promises to advance the identification of the factors and pathways crucial for healthy child development. Ultimately, this should contribute to better prevention and healthcare programs in the future, providing children the best possible start in life.

2. Paper I

Dieckmann, L., Lahti-Pulkkinen, M., Kvist, T., Lahti, J., DeWitt, P. E., Cruceanu, C., Laivuori, H., Sammallahti, S., Villa, P. M., Suomalainen-Konig, S., Eriksson, J. G., Kajantie, E., Raikkonen, K., Binder, E. B., & Czamara, D. (2021). Characteristics of epigenetic aging across gestational and perinatal tissues. *Clin Epigenetics, 13*(1), 97. https://doi.org/10.1186/s13148-021-01080-y

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Clinical Epigenetics

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Characteristics of epigenetic aging across gestational and perinatal tissues

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Abstract

Background: Epigenetic clocks have been used to indicate differences in biological states between individuals of same chronological age. However, so far, only few studies have examined epigenetic aging in newborns—especially regarding different gestational or perinatal tissues. In this study, we investigated which birth- and pregnancy-related variables are most important in predicting gestational epigenetic age acceleration or deceleration (i.e., the deviation between gestational epigenetic age estimated from the DNA methylome and chronological gestational age) in chorionic villus, placenta and cord blood tissues from two independent study cohorts (ITU, n = 639 and PREDO, n = 966). We further characterized the correspondence of epigenetic age deviations between these tissues.

Results: Among the most predictive factors of epigenetic age deviations in single tissues were child sex, birth length, maternal smoking during pregnancy, maternal mental disorders until childbirth, delivery mode and parity. However, the specific factors related to epigenetic age deviation and the direction of association differed across tissues. In individuals with samples available from more than one tissue, relative epigenetic age deviations were not correlated across tissues.

Conclusion: Gestational epigenetic age acceleration or deceleration was not related to more favorable or unfavorable factors in one direction in the investigated tissues, and the relative epigenetic age differed between tissues of the same person. This indicates that epigenetic age deviations associate with distinct, tissue specific, factors during the gestational and perinatal period. Our findings suggest that the epigenetic age of the newborn should be seen as a characteristic of a specific tissue, and less as a general characteristic of the child itself.

Keywords: Epigenetic clocks, Early development, Epigenetic age, Perinatal tissues, Cord blood, Placenta, Chorionic villi

Background

DNA methylation (DNAm) is considered a biomarker of aging, with the potential to uncover differences in the biological age between individuals of the same chronological age [1, 2]. Epigenetic clocks make use of individual methylation patterns to estimate epigenetic age, and deviations between chronological and epigenetic age can be used to calculate relative epigenetic age acceleration (epigenetic age older than chronological age) and epigenetic age deceleration (epigenetic age younger than chronological age) in underlying tissues [3–5]. Commonly, these measures of epigenetic aging are calculated as the residuals of regressing predicted epigenetic age on chronological age, also called epigenetic age acceleration residuals (EAAR).

Epigenetic age acceleration has been linked to differences in long-term health outcomes and all-cause

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mortality in adults [6–8]. Changes in DNA methylation status have been proposed to be a mechanism by which environmental influences may become biologically embedded [9–11], and in fact, epigenetic age has been shown to be moderated by environmental exposures and lifestyle risk factors, such as education, body mass index (BMI), nutrition and smoking, among others [12–14]. These findings underscore the utility of epigenetic clocks as a means to investigate aging processes in general, and how these relate to environmental exposures and negative health outcomes or diseases. However, despite the sensitivity to and importance of epigenetic programming during the early developmental period [15, 16], studies investigating epigenetic age during the earliest phase of life are still underrepresented.

Various epigenetic clocks have been developed, for different tissues, ages and purposes [7, 17–19]. Specifically for the gestational period, two clocks for cord blood [20, 21], as well as two clocks for placental tissue [22, 23] have been established. For gestational epigenetic age estimation in cord blood, both the Knight [20] and Bohlin [21] clocks have been used in previous studies. Applying Knight's clock, epigenetic age deceleration has been linked to exposure to negative pregnancy environments including insulin-treated gestational diabetes mellitus in a previous pregnancy, maternal history of depression and greater antenatal depressive symptoms, maternal Sjögren's syndrome and a prenatal adverse environment assessed with the cerebroplacental ratio, as well as negative prospective child outcomes such as early childhood psychiatric problems [24–26]. These findings, together with the observation that epigenetic age acceleration was related to a lower need of respiratory interventions, led to the hypothesis that gestational epigenetic age deceleration may be related to a lower developmental maturity [27]. This seems to be supported by results from the Bohlin clock, where epigenetic age acceleration has been associated with higher birth weight and length [28], as well as higher head circumference, vaginal delivery, male sex and higher maternal pre-pregnancy BMI [29]. However, epigenetic age acceleration has also been associated with lower birth length, a lower 1-min Apgar score, fetal demise in a previous pregnancy, maternal preeclampsia, maternal age over 40 years at delivery and treatment with antenatal betamethasone [24], thus not supporting this hypothesis. Despite that, it should be noted that it was recently shown that CpGs relevant for epigenetic aging in general were linked to developmental processes [30].

Regarding placental tissue, Mayne et al. [23] found epigenetic age acceleration to be associated with early onset preeclampsia. Another study using Mayne's clock reported a link between higher epigenetic age acceleration in the placenta and lower fetal weight and other growth measures among males, but increased fetal weight and growth among females [31]. Furthermore, placental epigenetic age deceleration has been associated with maternal weight gain during pregnancy, and for mothers of male offspring with pre-pregnancy obesity and higher blood pressure [32]. So far, to our knowledge, no comparable studies were performed with the placental clock presented by Lee [22]. Although research in this field is growing since the development of perinatal tissue clocks, studies considering different available clocks, and various birth- and pregnancy-related variables in a combined fashion, are largely lacking. More studies are needed to achieve a better understanding of the associations of epigenetic age deviations in perinatal tissues with exposures and outcomes, and especially how these deviations compare across tissues. Such insights are critical to gain a better knowledge of aging and developmental processes during the earliest phase in life and may help to find intervention strategies in the long term.

The aim of this explorative study was to I) identify factors among various birth- and pregnancy-related variables which are most predictive of epigenetic (DNAm) age acceleration or deceleration in first trimester placental tissue derived from chorionic villus sampling (CVS), term placenta and cord blood collected at birth, and II) characterize the relationship between epigenetic age deviations across gestational and perinatal tissues from the same individuals.

We used data from two independent Finnish cohorts, the intrauterine sampling in early pregnancy study (ITU), and the prediction and prevention of preeclampsia and intrauterine growth restriction study (PREDO). We assessed gestational epigenetic age in early-pregnancy CVS samples, and cord blood and fetal-side or decidual-side placental tissue sampled at birth (ITU: 693 individuals and 1176 tissue samples from CVS and/or term fetal placenta and/or cord blood, PREDO: 966 individuals and 1083 samples from term decidual placenta and/or cord blood). We calculated the epigenetic age with both available clocks per tissue, and applied Bohlin's clock for cord blood [21] and Lee's clock for placenta [22], based on better accuracy metrics of these clocks in the data sets. The predictive power of several birth- and pregnancy-related variables for a higher or lower deviance between estimated epigenetic and chronological gestational age (GA) was tested in every tissue separately, and finally, cross-tissue correlations were evaluated.

To the best of our knowledge, this is the first study of epigenetic age in CVS samples, and across multiple gestational/perinatal tissues assessed from the same individuals.

Methods

Study populations

The intrauterine sampling in early pregnancy study (ITU) consists of Finnish women and their children born between 2012 and 2017. The women were recruited through the national voluntary prenatal screening program for trisomy 21, available for all pregnant women in Finland free of charge.

ITU study comprises two study arms. 1) Women in the chromosomal testing arm had been referred to the Helsinki and Uusimaa Hospital District Fetomaternal Medical Center (FMC) because they had an increased risk of fetal chromosomal abnormalities based on routine serum and ultrasound screening, age, and patient history. They underwent fetal chromosomal testing (CVS, amniocentesis, or noninvasive prenatal testing) at FMC. Women were informed about the study during FMC visits. If the chromosomal test indicated no fetal chromosomal abnormalities, those who had expressed interest in participating were contacted for final recruitment. Those whose chromosomal test results suggested a fetal chromosomal abnormality were not recruited. 2) Women in the no chromosomal testing arm underwent the same routine screening for fetal chromosomal abnormalities. Based on their serum and ultrasound screening, age and patient history, they were *not* referred to FMC for fetal chromosomal testing. The women were informed about ITU when attending the routine screening at maternity clinics. Women who expressed interest in participating were contacted for final recruitment into this study. Both study arms provided placenta and cord blood samples for this study. CVS tissue was only acquired from the chromosomal testing arm participants who underwent CVS sampling at FMC.

The Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO) study is a longitudinal multicenter pregnancy cohort study of Finnish women and their singleton, born-alive children between 2006 and 2010 [33]. The recruitment took place when the mothers attended their first ultrasound screening in early pregnancy. The PREDO comprises two subsamples: the clinical arm recruited based on having risk factors for preeclampsia and intrauterine growth restriction, and the epidemiological arm recruited from study hospitals independently of the presence of risk factors.

All participating women in both cohorts signed written informed consent forms for them and their children to participate in the study. The consents enabled linkage of nationwide health register data using unique personal identification numbers assigned to all Finnish citizens and permanent residents since 1971.

Sampling of biological tissues

In ITU, CVS samples were taken based on medical indication between 10–15 weeks of gestation. Any CVS surplus tissue, not needed for clinical purposes, was immediately stored at − 80℃.

Placenta samples were collected after birth and midwives/trained staff took nine-site biopsies (within maximum 120 min after delivery for ITU, and maximum 90 min after delivery for PREDO). In ITU, placental samples were taken from the fetal side of the placenta, at 2–3 cm from umbilical cord insertion and the biopsies were first stored at + 5 °C and then at − 80 °C. In PREDO, samples were taken from the decidual side of the placenta and immediately stored at − 80 °C.

For both ITU and PREDO, cord blood samples were taken immediately after birth by a midwife.

DNA methylation

From the collected samples, DNA was extracted according to standard procedures. Methylation analyses were performed at the Max Planck Institute of Psychiatry in Munich, Germany. We aimed to use 400 ng DNA for bisulfite-conversion with the EZ-96 DNA Methylation kit (Zymo Research, Irvine, CA). For n = 71 CVS samples, this was not feasible and we used lower amounts of DNA (from 48 ng upward). We saw no relation between the amount of DNA and our quality control measures. DNA samples were run on the Illumina Infinium MethylationEPIC array (Illumina, San Diego, USA), and for an additional set of cord blood samples from PREDO on the Infinium HumanMethylation450 BeadChip (Illumina, San Diego, USA). In total, methylation levels were assessed in n = 277 CVS samples, n = 500 placental samples and n = 437 cord blood samples from ITU (all assessed on the EPIC array), and in n = 140 placental samples and n = 160 cord blood samples (EPIC array) and an additional n = 876 cord blood samples processed with the 450 K array from PREDO.

Preprocessing of all methylation samples was conducted using the same pipeline [34] and the R package *minfi* [35]. Scan intensity signals as stored in.idat files were loaded into R and transformed into beta-values.

Samples with a mean detection p value > 0.05 were excluded (ITU: eight for CVS, one for placenta, none for cord blood; PREDO: none for placenta, three for cord blood run on EPIC, three for cord blood run on 450 K). Additionally, we excluded samples presenting with distribution artifacts in raw beta-values (ITU: five for CVS, nine for placenta, one for cord blood; PREDO: none for placenta, three for cord blood run on EPIC, eight for cord blood run on 450 K), as well as samples showing sex mismatches between estimated sex (using the *getSex*

function) from methylation data and confirmed phenotypic sex (ITU: none for CVS, four for placenta, one for cord blood; PREDO: one for placenta, four for cord blood run on EPIC, n = 19 for cord blood run on 450 K). Further n = 20 samples needed to be excluded from the PREDO cord blood data set run on the 450 K array due to technical artifacts. Beta-values were normalized using stratified quantile normalization [36], followed by BMIQ [37]. Afterward, beta-values were transformed into M values, and batch-effects were removed using Combat [38]. For this, we computed a principal component analysis (PCA) on the M values and checked which batches were most strongly associated with the principal components. The strongest batches for the respective data set were iteratively removed (for ITU these were plate, array and slide in CVS; plate, slide and array in placenta; and plate and array in cord blood; for PREDO these were plate, array and slide in placenta; plate and array in cord blood run on the EPIC array; plate and array in cord blood run on the 450 K array). Corrected M values were re-transformed into beta-values.

In a next step, we applied MixupMapper [39] to the genotype and methylation data to check for possible sample mix-ups. Mix-ups occurred solely in the PREDO cord blood data set from 450 K array and n = 12 samples were removed.

For cord blood samples, contamination with maternal blood was tested [40] and samples identified as contaminated were excluded from further analyses (ITU: nine for cord blood; PREDO: one for cord blood run on EPIC, n = 19 for cord blood run on 450 K).

The final data sets from ITU comprise 264 samples from CVS, 486 samples from placenta and 426 samples from cord blood. The final data sets from PREDO comprise 139 samples from placenta, 149 samples from cord blood from EPIC and 795 samples from cord blood from 450 K.

The final data sets with sample sizes are illustrated in Fig. 1.

Gestational epigenetic and chronological age

Gestational epigenetic age (DNAm GA) was estimated for cord blood using both the methods proposed by Knight et al. [20] and Bohlin et al. [21]. For Knight's clock, the estimation of DNAm GA was based on the methylation profile of 142 from the original 148 CpGs, due to the lack of 6 CpGs on the EPIC array. Excluding the missing CpGs from the calculation was also recommended by the authors [20], who reported a high correlation between estimates from the full and reduced epigenetic age predictor. For the calculation of DNAm GA with Knight's clock, we applied the script provided by the authors on the raw, un-normalized data. For Bohlin's clock, the estimation of DNAm GA was constituted on 88 from 96 CpGs, also following from differences between the underlying arrays. DNAm GA in chorionic villi and placenta samples was estimated using 558 CpGs proposed by Lee et al. [22]. Additionally, we estimated DNAm GA using 57 CpGs available on the EPIC array from the original 62 CpGs determined by Mayne et al. [23]. A list of the CpGs missing on the EPIC array for the respective clocks can be obtained from Additional file 1.

Child chronological gestational age (GA) was based on fetal ultrasound, performed before 24 + 0 weeks of gestation and extracted from the Finnish Medical Birth Register (MBR).

Cell-type composition estimations

Cell-type composition into seven cell types (nucleated red blood cells, granulocytes, monocytes, natural killer cells, B cells, CD4(+) T cells and CD8(+) T cells) in cord blood was estimated in *minfi* based on the approach proposed in Gervin, Salas [41].

Cell-type composition into six cell types (nucleated red blood cells, trophoblasts, syncytiotrophoblasts, stromal, Hofbauer, endothelial) in CVS and placenta was estimated using a recently published reference [42] and implementation within the R package *planet*, by applying the robust partial correlation algorithm [43].

The mean estimated cell types for every data set are given in Additional file 2.

Genotyping and ancestry-related information

Genotyping was performed on Illumina GSA-24v2-0_A1 arrays for ITU, and on Illumina Human Omni Express Arrays for PREDO, according to the manufacturer's guidelines (Illumina Inc., San Diego, CA). Quality control was performed in Plink 1.9 [44] and R [45]. DNA was extracted from cord blood, if available, otherwise placental tissue was used. SNPs with a minor allele frequency below 1%, a call rate below 98%, or with deviation from Hardy–Weinberg-Equilibrium with a p value $< 1 \times 10^{-05}$ were removed from the analysis. Furthermore, SNPs mapping to multiple locations as well as duplicated variants were removed. Individuals with a genotype call-rate below 98% were also excluded. Any pair of samples with IBD estimates > 0.125 was checked for relatedness. Within PREDO, high IBD estimates could be resolved due to shared ethnical origin of these individuals except for one pair. From this pair, we excluded one sample from further analysis. In ITU, seven samples were removed. Furthermore, individuals showing discrepancies between phenotypic and genotypic sex (one in PREDO, none in ITU) were removed.

To retrieve ancestry-related information, we performed multi-dimensional scaling (MDS) analysis on the IBS

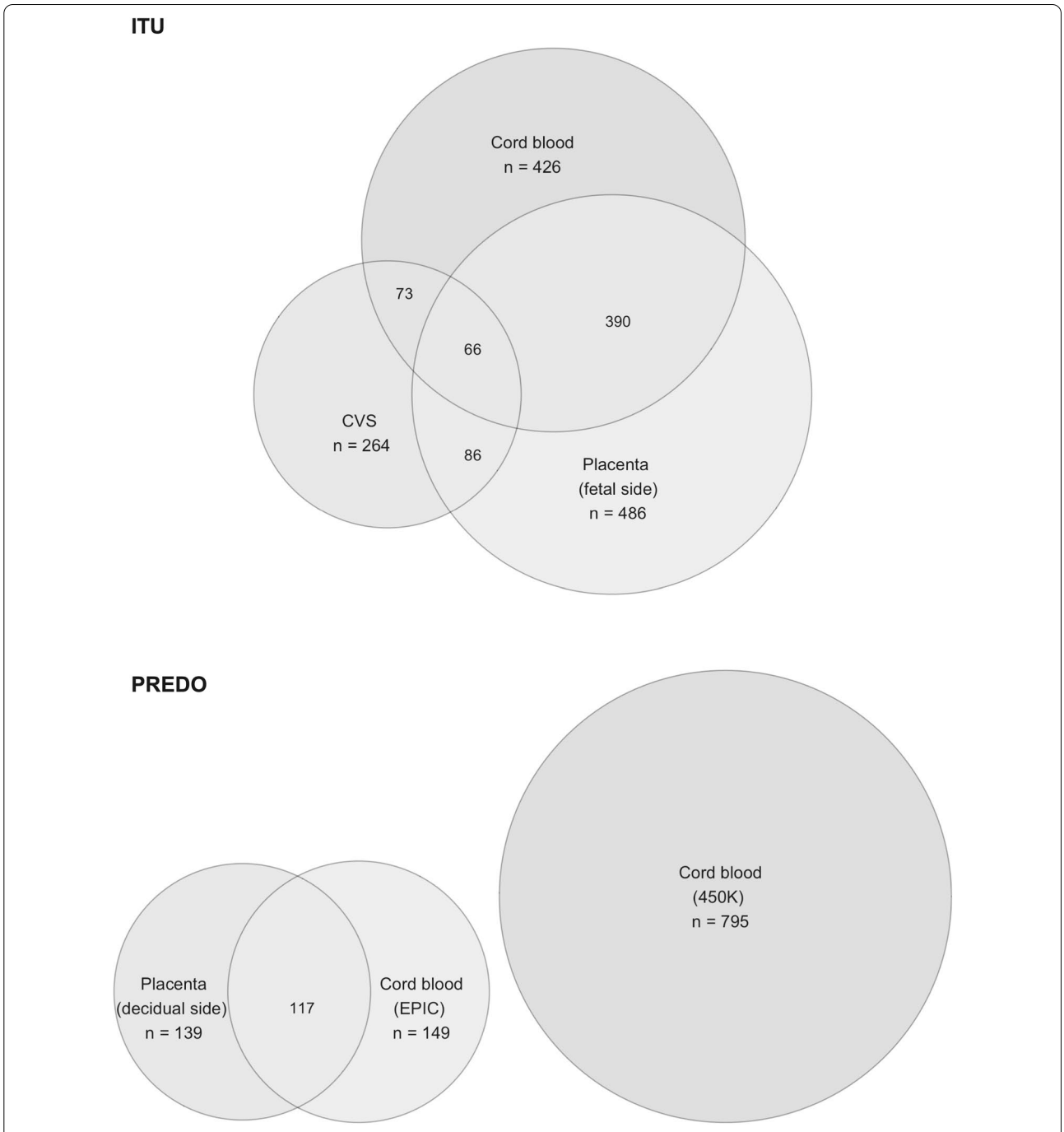

Fig. 1 Sample overview for both cohorts used. Samples with methylation data available from different tissues in ITU and PREDO. In total, the ITU data set comprised 693 individuals after QC, with 264 CVS, 486 fetal placenta and 426 cord blood samples. For some individuals, samples were available from several tissues, indicated by overlapping circles. The final PREDO data set comprised 171 individuals after QC processed with the EPIC array, and additional 795 individuals processed with the 450 K array. From the EPIC data, 139 samples were available from placenta, and 149 samples from cord blood. The number of individuals with data from both tissues is again illustrated by the overlapping circles

matrix of quality-controlled genotypes [46], where available. Outliers, defined as samples presenting with a position on any of the first ten axes of variation deviating more than four standard deviations from the respective axis' mean, were iteratively removed until no more outliers were detected. Afterward, individuals presenting with heterozygosity values more than four standard deviations away from the mean heterozygosity were also iteratively

removed (none in PREDO, two in ITU). The first two components were extracted and included as covariates in following analyses. In total, ancestry-related information for ITU was available from 587 of the 693 individuals included in our analyses, for 148 of the 171 individuals from PREDO with methylation data from the EPIC array, and for 787 of the 795 individuals from PREDO with methylation data from the 450 K array.

Birth- and pregnancy-related variables

We included 14 birth- and pregnancy-related variables which were available for all tissues in both data sets.

In both cohorts, child sex, birth weight (kg), birth length (cm) and birth head circumference (cm) were measured at birth and data were extracted from the MBR. Maternal age (years) at delivery, early pregnancy BMI, calculated from weight and height verified by measurement at the first antenatal clinic visit, smoking during pregnancy (yes or no), parity (primiparous or multiparous), mode of delivery (unaided vaginal delivery or aided delivery, including breech, forceps, vacuum, cesarean section), and induction of labor (yes or no) were obtained from the MBR. Diagnoses of maternal diabetes disorders (yes for both types I & II, as well as gestational diabetes [ICD-10: E08-E14, O24] or none) until childbirth, and hypertensive pregnancy disorders such as gestational hypertension or pre-eclampsia in the current pregnancy (yes [ICD-10: O10-O14] or no), were extracted combining data from the MBR and the Finnish nationwide Care Register for Healthcare (CRHC). The CRHC carries primary and subsidiary diagnoses of all inpatient and outpatient hospital visits in Finland and from all treatments in specialized public outpatient care in Finland. In PREDO, the CRHC and MBR diagnoses were confirmed by a clinical jury, which comprised two physicians and a study nurse. Diagnoses of any maternal mental or behavioral disorder [ICD-8 and ICD-9: 290–319; ICD-10: F00-F99] until child birth were extracted from the CRHC. Alcohol use during early pregnancy was reported by the mothers (for PREDO around gestational week 12–13, for ITU around gestational week 20).

Statistical analyses

All statistical analyses were conducted in R version 4.0.2 [45].

Measuring deviations between epigenetic age and chronological age

In previous studies, two measures of epigenetic age acceleration were considered, one based on the raw difference between DNAm age and chronological age, and the other calculated as the residuals from regressing DNAm age on chronological age. While the former provides a more intuitive interpretation and the investigation of the disjunct effects of epigenetic age, the latter is preferable in terms of its statistical properties—it addresses the dependency of age acceleration on chronological age and is comparable across studies. Therefore, we defined the deviation between gestational epigenetic age (DNAm GA) and chronological gestational age (GA) in all statistical models as the residuals (*epigenetic age acceleration residuals, EAAR*) resulting from regressing DNAm GA on GA, cell types of the respective tissue and the first two ancestry-related components derived from genotypic information. A positive EAAR value suggests faster biological aging, i.e., a higher epigenetic than chronological age (epigenetic age acceleration), and a negative EAAR value suggests slower biological aging, i.e., a lower epigenetic than chronological age (epigenetic age deceleration).

Identification of factors impacting epigenetic age acceleration/deceleration per tissue

Our aim was to identify those of the available birth- and pregnancy-related variables that were most predictive of higher or lower EAAR. Without sufficient prior information enabling a hypothesis-driven selection of predictors, we decided to choose an appropriate data-driven variable selection method. Further, to reduce confounding effects, all predictors were evaluated in one model and correlations between predictors (see Fig. 2 for an overview) were considered, using elastic net regressions combined with a bootstrap approach for an evaluation of robustness. Separate models were run for all tissues, and cohorts, including placental models from ITU (fetal) and PREDO (decidual).

For every model, all predictor variables and the outcome variable (EAAR) were z-standardized, to ensure that the penalization was fair to all regressors and to enable the interpretation of the size of coefficients in terms of importance. Further, only complete observations were included. Bootstrapping was performed with 1000 bootstrap samples on every input data set. On every bootstrap sample, an elastic net regression was fitted with the R package *ensr* [47], which is built on *glment* [48]. Hyperparameters were selected by tenfold cross-validation, default lambda values (n = 100) and a sequence of 11 alpha values between 0 and 1 (by steps of 0.1). The output was grouped by bootstrap and number of non-zero coefficients (nzero) resulting from the different alpha levels. Of these, the models with minimum mean cross-validation error (cvm) with the respective parameters were chosen as best models (for every bootstrap and number of nzero). Afterwards, the percentage of a variable being not zero was calculated over all bootstrap samples for every number of nzero. Further, the median cvm over the

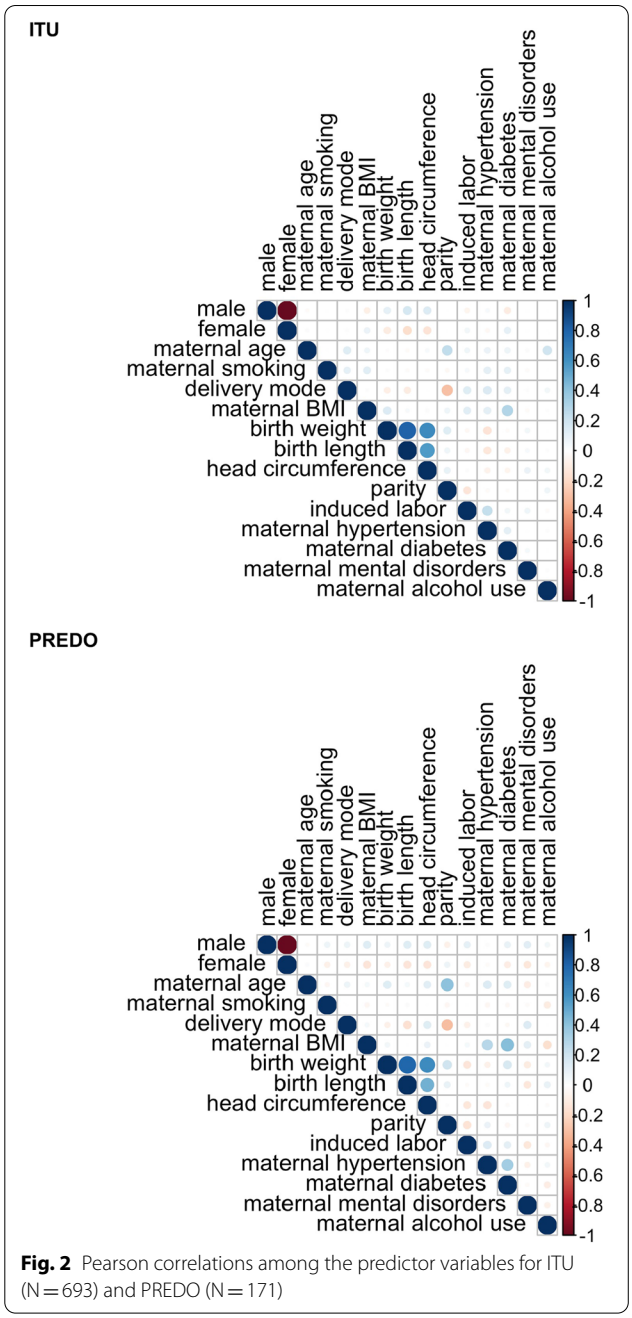

Fig. 2 Pearson correlations among the predictor variables for ITU (N = 693) and PREDO (N = 171)

bootstrap samples was plotted for every number of nzero. At this point, a final number of nzero must be chosen, with a necessary trade-off between model complexity and error (bias-variance tradeoff [49]). To aid the decision of non-zero coefficients in smoothly decreasing curves, we looked at the elbow in the plot of the median cvm for every nzero, by using a function drawing a straight line from the first to the last point of the curve and finding the data point farthest away from this line. This point can indicate the position of most decreasing cvm. The respective number of nzero can be used for further analysis steps. Due to the bootstrapping procedure, there was still variation in the variables and their coefficients in the final model. If a predictor was selected in > 75% of the bootstrap samples, we declared it as sufficiently stable and important. This approach for variable selection was referred to as *variable inclusion probability (VIP)* in a previous paper, where the authors used a comparable method for neuroimaging data [50]. The median coefficients and 95% confidence intervals over bootstraps, when the variable was not zero, were also calculated. An illustration of the analysis steps is given in Additional file 3.

Replication of cord blood findings between cohorts

To evaluate the predictability of the chosen predictors in ITU in the PREDO data set, the median coefficients of the identified variables in ITU were used to predict EAAR in PREDO. The one-tailed Pearson correlation between predicted and observed EAAR values was calculated. Additionally, we performed the same elastic net analysis applied in ITU cord blood data independently in the PREDO cord blood data sets to confirm that the directions of associations are consistent with those observed in ITU (Additional file 4).

Cross-tissue analyses

Pearson correlations for both DNAm GA and EAAR were calculated between cord blood and placenta, as well as between CVS and placenta and CVS and cord blood, for persons with multiple tissue sample available. To test if there are significant differences in mean age acceleration or deceleration between the tissues, we applied paired Student's t tests, or paired Wilcoxon signed-rank test, between EAAR values of the respective tissues.

Complementary analyses

It has been reported that child sex can be an important factor when considering how placenta function is affected by direct environmental factors [51], and sex differences in epigenetic aging have been reported [31, 32]. Therefore, we repeated our analyses in placenta stratified by sex as described in Additional file 5.

Additionally, information about maternal alcohol use during pregnancy was only available in 580 samples from ITU, 153 samples from the EPIC array in PREDO and 693 samples from the 450 K array in PREDO.

To avoid larger reductions in sample size for the remaining predictors, we did not include this variable in the main models per tissue, but provide it in supplementary analyses (Additional file 6).

Results

A summary of characteristics of the available data sets is given in Table 1.

Performance of epigenetic clocks in the investigated tissues

We first evaluated the performance of the two epigenetic clocks for cord blood [20, 21] and for placenta [22, 23] in our sample. The clocks differ in the included CpGs, and only share two CpGs (cg07816074, cg16536918; cord blood clocks) with negative weights, and one CpG (cg00307685; placenta clocks) with positive weight, respectively. Nevertheless, we observe high Pearson correlations in DNAm GA between the cord blood clocks ($r=0.77$, $p<0.001$ for ITU; $r=0.76$ $p<0.001$ for PREDO EPIC data; $r=0.51$, $p<0.001$ for PREDO 450 K data), and a medium to high Pearson correlation in the placenta clocks ($r=0.44$, $p<0.001$ for ITU; $r=0.48$, $p<0.001$ for PREDO). Scatter plots are provided in Additional file 7: Figure S5.

To evaluate the accuracy of an epigenetic clock, three main metrics have been proposed: the average difference between DNAm age and chronological age, the median absolute difference between DNAm age and chronological age, and the correlation between DNAm age and chronological age [4]. As shown in Table 2, the overall accuracy of the clocks was satisfactory, with relatively low median absolute deviations and high Pearson correlations between DNAm age and chronological age (see Additional file 7: Figure S6 for scatter plots). It is evident from these statistics that the estimations were more precise for cord blood as compared to placenta. Furthermore, Bohlin's clock outperformed Knight's clock for cord blood and Lee's clock outperformed Mayne's clock for placenta for all of the named criteria. Based on this, all following analyses were conducted with Bohlin's clock for cord blood and Lee's clock for placenta. Between these clocks, there is no overlap in the underlying CpGs.

Factors impacting the relative epigenetic age in gestational and perinatal tissues

The association between epigenetic age acceleration residuals (EAAR) and birth- and pregnancy-related variables was tested for cord blood, CVS and placenta tissue separately. The results of the elastic net regressions are summarized in Fig. 3, and further statistical parameters can be found in Additional file 9.

Analyses in cord blood

Cord blood in ITU Cord blood samples from ITU with full observations were available for 385 newborns. As described previously in the Methods section, nzero

Table 1 Characteristics of available data sets: *Mean (SD)* or *N (%)* for every variable

	ITU			PREDO		
	Cord blood	CVS	Placenta (fetal)	Cord blood (EPIC)	Cord blood (450 K)	Placenta (decidual)
Sample size	426	264	486	149	795	139
Gestational age (weeks)	40.04 (1.55)	12.79 (0.82)	39.99 (1.60)	39.87 (1.42)	39.74 (1.67)	39.89 (1.43)
Maternal alcohol use, yes[c]	40 (10)	24 (14)	48 (10)	16 (12)	115 (17)	17 (14)
Maternal smoking, yes[a,b]	18 (4)	29 (11)	20 (4)	13 (9)	32 (4)	13 (9)
Maternal mental disorders, yes	46 (11)	26 (9)	55 (11)	20 (14)	63 (8)	18 (13)
Maternal diabetes, yes[a, c]	93 (22)	57 (22)	105 (22)	26 (17)	222 (28)	20 (14)
Maternal hypertensive disorder, yes[a, b, c]	26 (6)	23 (9)	28 (6)	36 (24)	272 (34)	33 (24)
Maternal BMI[a, b, c]	23.94 (4.21)	24.20 (4.27)	23.82 (4.16)	25.23 (5.76)	27.38 (6.30)	24.85 (5.79)
Maternal age (years)[a, b, c]	34.70 (4.81)	34.96 (5.75)	34.59 (4.86)	32.13 (5.00)	33.33 (5.74)	32.04 (5.17)
Multiparous, yes[b, c]	193 (45)	153 (58)	235 (48)	85 (57)	558 (71)	74 (53)
Induced labor, yes	114 (27)	66 (25)	125 (26)	37 (25)	240 (30)	31 (22)
Delivery mode, aided[a]	129 (30)	87 (33)	145 (30)	51 (35)	233 (30)	55 (40)
Head circumference (cm)	35.10 (1.52)	35.04 (1.73)	35.07 (1.62)	35.21 (1.36)	35.13 (2.15)	35.19 (1.34)
Birth length (cm)[a, b]	50.23 (2.20)	50.13 (2.24)	50.17 (2.40)	49.77 (2.48)	50.21 (2.44)	49.65 (2.53)
Birth weight (g)[a]	3532 (489)	3489 (526)	3534 (509)	3454 (519)	3546 (559)	3425 (523)
Child sex, female	210 (49)	124 (47)	238 (49)	73 (49)	372 (47)	72 (52)

Differences in predictor variables between the ITU and PREDO data sets were tested using t tests for continuous variables and Chi^2 tests for categorical variables. Variables that showed nominal statistically significant differences ($p<.05$) are indicated as follows:

[a] For difference between ITU placenta vs. PREDO placenta data sets

[b] For difference between ITU cord blood vs. PREDO EPIC cord blood data sets

[c] For difference between ITU cord blood vs. PREDO 450 K cord blood data sets

Table 2 Performance metrics of the four clocks in all available tissues

Cord blood	**Bohlin's clock**						**Knight's clock**					
	DNAm GA		**Δ DNAm GA**			***r***	**DNAm GA**		**Δ DNAm GA**			***r***
	M	***SD***	***M***	***SD***	***MAD***		***M***	***SD***	***M***	***SD***	***MAD***	
ITU	39.80	0.93	− 0.23	0.94	0.92	.83*	38.91	1.47	− 1.13	1.19	1.17	.69*
PREDO (EPIC)	39.72	0.84	− 0.16	0.90	0.98	.80*	39.23	1.39	− 0.64	1.05	0.88	.72*
PREDO (450 K)	38.84	1.14	− 0.90	1.19	1.02	.70*	38.44	2.02	− 1.29	1.90	1.55	.48*
	Lee's clock						Mayne's clock					
	DNAm GA		Δ DNAm GA				DNAm GA		Δ DNAm GA			
Placenta	*M*	*SD*	*M*	*SD*	*MAD*	*r*	*M*	*SD*	*M*	*SD*	*MAD*	*r*
ITU CVS	10.55	1.48	− 2.24	1.14	1.07	.64*	11.69	1.81	− 1.09	1.63	1.57	.43*
ITU Placenta	38.53	1.40	− 1.45	1.41	1.29	.56*	32.68	1.91	− 7.31	1.91	1.73	.28*
PREDO	38.03	1.25	− 1.85	1.24	1.10	.58*	31.69	1.44	− 8.19	1.56	1.63	.41*

M = mean; *SD* = standard deviation; *MAD* = median absolute deviation; *r* = Pearson correlation coefficient for DNAm GA and chronological GA; DNAm GA = DNA methylation gestational age; Δ DNAm GA = raw difference between estimated DNA methylation gestational age and chronological gestational age (measured in weeks)

* $p < 0.001$

(number of non-zero coefficients) of the elastic net model was chosen by finding the most decreasing median cvm (minimum mean cross-validation error) across bootstrap samples. If a predictor was selected in > 75% of bootstrap samples in this model, we declared it as sufficiently stable. For cord blood data from ITU, the model was chosen with nzero = 9, and five variables were selected in a sufficiently stable manner: maternal smoking (97% of bootstrap samples), maternal mental disorders (83%), delivery mode (87%), birth length (95%) and female sex (84%). Maternal smoking, maternal mental disorders, aided delivery and higher birth length were associated with relatively higher EAAR; female sex was associated with relatively lower EAAR (see Fig. 3a).

Replication of cord blood findings in PREDO Cord blood data were available from both cohorts which enabled a test of the performance of these predictors identified in ITU in an independent cohort (PREDO). In PREDO, 144 samples had complete data from the EPIC array, and 766 from the 450 K array. The beta matrix of median coefficients derived from the final model in ITU was used for a prediction of EAAR in PREDO. The one-tailed Pearson correlation between predicted and true EAAR was $r = 0.24$, $p = 0.002$ for the EPIC array and $r = 0.11$, $p = 0.002$ for the 450 K array (Additional file 8: Fig. S7), supporting that the predictors of EAAR identified in the ITU cohort can be predictive for relative epigenetic age acceleration/deceleration in independent cohorts and different array platforms. We then further analyzed the PREDO data sets independently (Additional file 4) and compared the results with those from ITU. The direction of effects between the predictors and EAAR was consistent across cohorts; however, the strength of the associations and most predictive variables varied between data sets.

Analyses in placental tissues

CVS in ITU For CVS, 195 samples were available with full information for all predictor variables and EAAR. The elastic net regression model with nzero = 8 was chosen. Maternal smoking was the only variable with non-zero coefficients in more than 75% of the bootstrap models (81%), and associated with relatively higher EAAR (see Fig. 3b).

Placenta (fetal) in ITU For fetal placenta in ITU, 427 complete observations were available, and the model with nzero = 7 was chosen. In this model, three variables had non-zero coefficients in > 75% of the bootstrap models: Child sex (99%), parity (78%) and maternal mental disorders (82%). Maternal mental disorders were associated with relatively higher EAAR, while being multipara and female sex of the child were related to relatively lower EAAR (see Fig. 3c).

Placenta (decidual) in PREDO For decidual placenta, the model could be built from 117 samples, and nzero = 6 was selected. In this model, maternal mental disorders occurred sufficiently stably over the bootstrap samples (96%) and were associated with relatively lower EAAR (see Fig. 3d).

Complementary analyses

Separate analyses for male and female placentas are described in detail in Additional file 5. These analyses showed that the strength of association of predictors

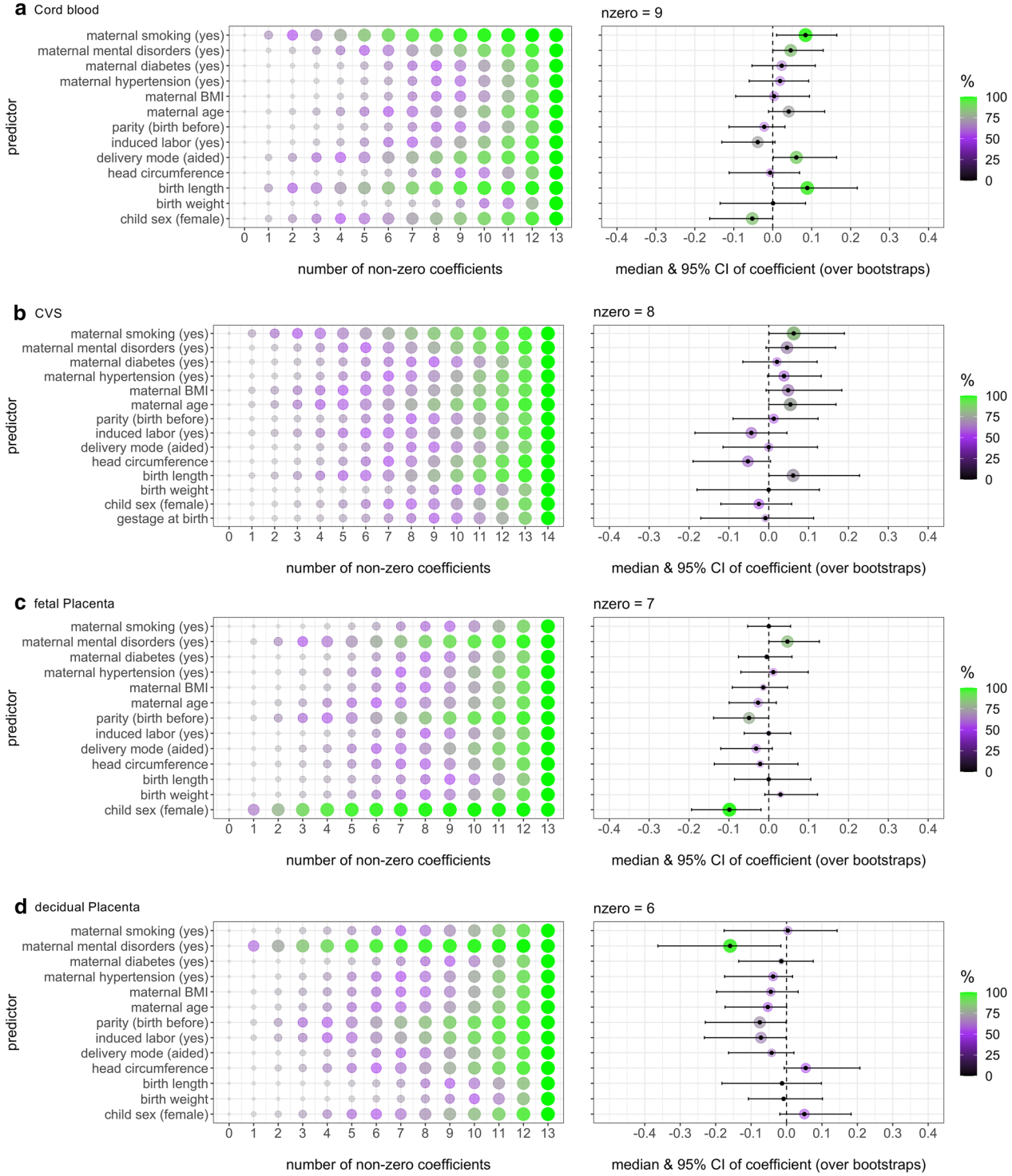

Fig. 3 Outcomes of elastic net regression models in different tissues. Associations between birth- and pregnancy-related variables (predictors) and EAAR (adjusted for gestational age at time of sampling, cell types and ancestry-related information). Depicted are the percentages of variable occurrence in bootstrap models with different number of non-zero coefficients (left) and the coefficients of variables in the final model (right) in cord blood from ITU (**a**), CVS from ITU (**b**), fetal placenta from ITU (**c**) and in decidual placenta from PREDO (**d**). The color coding shows the percentage of occurrence of a variable in the model over bootstraps and the size of the circle is proportional

with epigenetic age acceleration/deceleration can differ between males and females. Further, some predictors showed tendencies of different directions of associations between males and females, but as these patterns were not sufficiently stable and strong in our analyses, this needs to be confirmed with larger sample sizes in future studies.

We additionally report analyses including maternal alcohol use (smaller sample sizes, n = 367 in cord blood, n = 133 in CVS, n = 412 in placenta from fetal side (ITU), and n = 106 in placenta from decidual side (PREDO)) in Additional file 6. Overall, maternal alcohol use does not seem to be strongly related to epigenetic age acceleration or deceleration in gestational and perinatal tissues; only a weak association was found with relatively higher EAAR in decidual placenta.

Cross-tissue analyses

To evaluate how epigenetic age and acceleration or deceleration relate between the tissues, we calculated Pearson correlations between the DNAm GAs and EAARs, respectively. We further tested for statistically significant differences in epigenetic age acceleration/deceleration between tissues using paired Student's t test or paired Wilcoxon signed-rank test in case of unfulfilled assumptions for the parametric test. We illustrate the differences in EAARs between tissues from the same individuals in Fig. 4. For n = 60 children from ITU with complete tissue data (cord blood, CVS and fetal placenta), we illustrate individual differences in EAAR in Fig. 4d.

Cord blood and placenta

The correlation between DNAm GAs of cord blood and placenta was significant in both ITU, $r = 0.48$, $p < 0.001$, and PREDO, $r = 0.48$, $p < 0.001$. This was expected, as the DNAm GA is an estimator of GA, which is the same for these tissues at birth. However, there was no significant correlation between the EAARs, neither in ITU, $r = -0.03$, $p = 0.53$, nor in PREDO, $r = 0.09$, $p = 0.32$ (Fig. 4a). This suggests that individual epigenetic age acceleration does not correspond between cord blood and fetal placenta, nor between cord blood and decidual placenta. Furthermore, there was no indication of generally higher or lower age acceleration/deceleration in cord blood ($M = -0.01$, $SD = 0.49$) and fetal placenta ($M = -0.02$, $SD = 1.11$) from ITU, $t = 0.16$, $p = 0.88$, nor in cord blood ($M = -0.01$, $SD = 0.48$) and decidual placenta ($M = 0.01$, $SD = 0.90$) from PREDO, $t = -0.27$, $p = 0.79$.

CVS and (fetal) placenta

The correlation between DNAm GAs of CVS (at sampling) and fetal term placenta in ITU was significant $r = 0.27$ $p = 0.01$. However, there was no significant correlation between the EAARs at sampling in CVS and fetal placenta, $r = 0.18$, $p = 0.11$ (see also Fig. 4b). Overall, epigenetic age acceleration/deceleration was not significantly higher or lower in CVS ($M = 0.03$, $SD = 0.93$) versus fetal placenta ($M = 0.14$, $SD = 1.0$), $t = -0.73$, $p = 0.47$.

CVS and cord blood

Neither the correlation between DNAm GAs of CVS and cord blood in ITU $r = 0.09$, $p = 0.46$, nor the correlation between the EAARs at sampling in CVS and cord blood, $r = 0.12$, $p = 0.34$ was significant (see Fig. 4c). Paired Wilcoxon signed-rank test showed no significant difference in epigenetic age acceleration/deceleration between CVS ($M = 0.08$, $SD = 0.95$) and cord blood ($M = -0.07$, $SD = 0.54$), $p = 0.32$.

Discussion

Our analyses uncovered the strength and direction of associations between several birth- and pregnancy-related variables with gestational epigenetic age acceleration or deceleration in CVS, cord blood, fetal and decidual placenta tissue. Further, we showed that the factors related to epigenetic aging differ between the tissues, and that there is no correspondence in individual epigenetic age deviations across these tissues.

Insights from single tissue analyses

We will first discuss variables that showed associations with epigenetic age deviations. Among the considered child characteristics, we found newborn anthropometric data, especially birth length, to be associated with relatively higher epigenetic age acceleration in cord blood. This is in accordance with two other studies applying Bohlin's clock [28, 29]. In contrast, anthropometric characteristics of the child seem to be less associated with epigenetic aging in placental tissues. Female child sex was related to relatively lower epigenetic age acceleration in both cord blood and fetal placenta.

Regarding maternal characteristics, smoking during pregnancy was associated with relatively higher epigenetic age acceleration. We observed this in cord blood as well as CVS tissue, but neither in fetal, nor decidual term placenta.

Furthermore, maternal mental disorders showed an association with epigenetic age acceleration in cord blood and in fetal placenta within the ITU cohort. However, in PREDO, maternal mental health disorders were not associated with cord blood epigenetic age, but these disorders

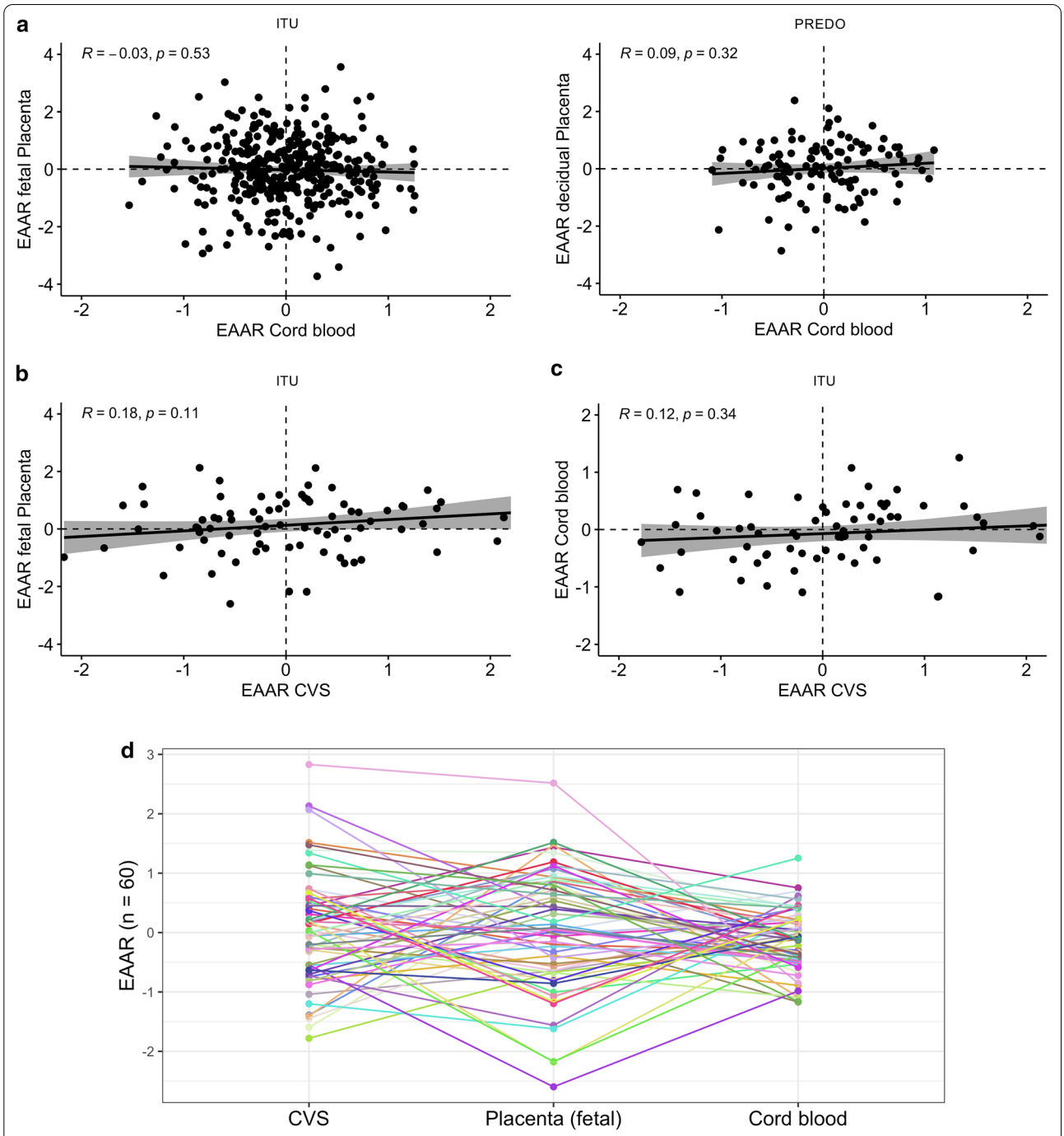

Fig. 4 Relationship of epigenetic age acceleration/deceleration between different tissues. In children with more than one tissue available, the relationship of epigenetic age acceleration or deceleration between the respective tissues can be illustrated. Depicted are the scatter plots of EAAR for (**a**) cord blood and placenta from both ITU (n = 363) and PREDO (n = 116), (**b**) CVS and placenta from ITU (n = 78), and (**c**) CVS and cord blood from ITU (n = 66). The regression line is plotted together with a 95% confidence interval, and the Pearson correlation coefficient is depicted. Individual differences in EAARs between CVS, placenta and cord blood from ITU are further illustrated (**d**) for n = 60 children from ITU, where each color represents one individual.

were associated with epigenetic age deceleration in decidual placenta. Medical treatment can be of relevance when considering mental diagnoses, for example, the influence of considering SSRIs was reported in a previous study [52]. However, the differences between ITU and PREDO are unlikely to be due to differences in the

prevalence or treatment of mental disorders between the samples: the rate of mental disorders was similar across samples and both cohorts had similar access to care through the Finnish healthcare system. In both cohorts, lifetime occurrence of any mental disorder up to childbirth was identified in the same way based on national register data. Nevertheless, some differences between the cohorts remain: while PREDO was enriched for participants with risk factors of pre-eclampsia and intrauterine growth restriction, ITU was enriched for participants who underwent prenatal fetal chromosomal testing. It is possible that these differences in the populations explain some discrepancies between the findings. Furthermore, differences in epigenetic aging may also arise from distinct biological characteristics of the two placental regions with different functions and tissue composition. In fact, cell count estimates between CVS and placenta but also between fetal and decidual placenta showed substantial differences (see Additional file 2). Altogether, our results support the hypothesis that maternal mental disorders associate with epigenetic age deviations in perinatal tissues. We encourage future studies, e.g. with both decidual- and fetal-side samples from the same individuals, to further evaluate tissue specificity.

Another predictor related to the mother and pregnancy was parity, which showed an association with epigenetic age deceleration in fetal placenta. Out of the variables related to the delivery process itself, aided delivery was associated with relatively higher epigenetic age acceleration in cord blood.

Overall, relevant predictors for relative epigenetic age acceleration in gestational and perinatal tissues span the whole spectrum from child and mother to birth and pregnancy characteristics.

Our results indicate that relatively lower or higher epigenetic age deviation cannot be clearly assigned to birth- and pregnancy-related variables that are considered as being more favorable versus unfavorable in the context of disease risk. This suggests that gestational epigenetic age acceleration or deceleration itself may not be linked to a higher risk for diseases per se, but that these associations are more complex and dependent on the condition and tissue during the earliest phase of life. It has been proposed that adjustments to the maturational tempo may explain why children in both favorable and unfavorable environments can exhibit epigenetic age acceleration, as this possibly constitutes specific adaptations to future challenges [53, 54]. Recent studies in adult populations also reported large differences in associations with lifestyle risk factors among studies and clocks [14, 55, 56], and it was assumed that different epigenetic clocks may capture the consequences of different environmental stimuli [14]. Overall, it has to be noted that the mechanistic underpinnings of biological age and epigenetic clocks are still discussed and not fully understood [3, 19, 54].

Cross-tissue relationships

In addition to looking at factors associated with gestational epigenetic aging in single tissues, we investigated the epigenetic age relationship between tissues. The estimated epigenetic age was congruent between cord blood and placenta, which has also been reported for most tissues investigated in adults so far with only few exceptions [7].

There was no evidence for one tissue being in general epigenetically older or showing remarkable biases toward epigenetic age acceleration or deceleration. However, the relative epigenetic age acceleration or deceleration in the different tissues was not concordant, i.e. a child with relatively high EAAR in one tissue did not necessarily display relatively high EAAR in another tissue (see Fig. 4d). This is in accordance with the fact that we observed different predictors as being the most related to epigenetic aging in the different tissues, and in line with the proposition of different characteristics of epigenetic age acceleration between diverse tissues [19, 57]. Although we can only speculate about the underlying processes at this point, these results suggest that the factors with strongest influence on gestational epigenetic age acceleration and deceleration vary between functionally different parts of one tissue (fetal vs. decidual placenta), developmental stage of the placenta (CVS vs. term placenta), and between placental and cord blood tissues. This indicates that with the currently available epigenetic clocks for specific gestational/perinatal tissues, the epigenetic age of the newborn should be seen as a characteristic linked to the respective tissue, and less as a general characteristic of the child itself. Thus, future health and developmental trajectories associated with gestational epigenetic age can be expected to show a more tissue dependent pattern, too, which should be kept in mind when interpreting results from one tissue. It would be interesting to see if a cross-tissue or phenotypic clock for the gestational and perinatal period, as developed for adults [4, 58], also shows more congruent associations of epigenetic age acceleration and deceleration in newborns with different predictors and outcomes. However, it may also be that tissue-specific effects are generally more pronounced in gestational and perinatal tissues, probably because of the particularly dynamic (epigenetic) processes taking place in these tissues, and therefore especially important to consider and disentangle.

Strengths and limitations

A major strength of the present study is the inclusion of three different perinatal tissues. Insights into epigenetic age acceleration in CVS are unique, as well as the examination of epigenetic aging across gestational and perinatal tissues. In addition, we were able to compare and contrast tissues from two independent Finnish cohorts. While the context of recruitment for the two studies was different, as elaborated above, the individual predictors were comparable across studies and showed very similar correlation structure (see Table 1 and Fig. 2). To thoroughly assess the impact of the different factors and account for confounding, we chose a modeling approach that enables the inclusion of all variables in one model, can deal with correlations among predictors and performs variable selection [59]. We restricted the analysis to the set of variables which were available for both cohorts and all tissues. On the one hand, this is a strength, as this approach allowed us to identify important predictors of epigenetic aging (in cord blood) in one cohort, and then test these predictors in a second, independent, cohort, to validate the findings. These predictors are also likely to be available in many clinical settings and study cohorts. On the other hand, this approach has its limitations, as there are likely additional factors influencing gestational epigenetic age acceleration/deceleration, which were beyond the scope of the current study. Additional assessments of biological maternal variables, such as hormone levels, immune status and placental functional, could be important to better characterize influences on gestational epigenetic aging. Further, the presented results are of correlative nature, and we refer to perinatal factors as predictors even when they occurred after the measurement of the outcomes, which was done for consistency, modeling reasons and ease of interpretation, but does not imply a causal assumption. Studies in animal models or in vitro may help to better understand in which cases epigenetic age acceleration or deceleration is a cause versus consequence of other factors. Additionally, we did not include any postnatal measures in this analysis. Thus, future studies should test whether epigenetic age deviations in any of these tissues associate with altered health trajectories. Furthermore, investigating the relationship between genetic architecture and epigenetic aging during the gestational period was beyond the scope of the current analysis, but further studies incorporating similar approaches as already used for adults [60, 61] may also provide additional insights for the earliest developmental phase. Apart from this, both cohorts are of Finnish origin, which could reduce the generalizability of findings to other ethnicities and countries with, for example, lower socioeconomic status and prenatal health care, as well as for clinical samples. Despite the relatively large data resource, missing values led to a reduction of sample sizes, and biospecimens for more than one tissue were only available for a smaller proportion of individuals. When considering differences between fetal and decidual placenta, it is necessary to take into account that these samples were not only taken from different sides of the placenta, but also from different individuals and cohorts. Future studies sampling the same placenta from different sides are needed to better understand potential biological differences.

Conclusions

Our results suggest that factors affecting the deviation between gestational epigenetic and chronological age differ between gestational and perinatal tissues. In addition, more or less favorable birth- and pregnancy-characteristics were not associated with either accelerated or decelerated epigenetic age in a consistent direction. This indicates that both epigenetic age acceleration and deceleration are associated with distinct risk and protective factors, and possibly distinct, tissue specific, developmental trajectories in newborns. In line with this, there is no concordance between epigenetic age acceleration/deceleration in different gestational and perinatal tissues from the same individual. Overall, when using the currently available tissue specific clocks, the epigenetic age of the newborn should be evaluated on the tissue-level rather than on the individual level. Considering this can lead to important insights in health trajectories which may be distinct depending on the epigenetic aging profile of the underlying tissue.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-021-01080-y.

Additional file 1. Table S1: CpGs from the epigenetic clocks that were not included due to their missingness on the EPIC array.

Additional file 2. Table S2: Mean estimated cell-type proportions (%) and SD in every data set.

Additional file 3. Figure S1: Illustration of analysis steps using cord blood data from ITU.

Additional file 4. Supplementary analysis. Factors impacting the relative epigenetic age in cord blood from the PREDO cohort.

Additional file 5. Supplementary analysis. Factors impacting the relative epigenetic age in placenta, stratified by sex.

Additional file 6. Supplementary analysis. Factors impacting the relative epigenetic age, analyses including maternal alcohol use as predictor.

Additional file 7. Figures S5 and S6. Fig. S5: Scatter plots illustrating the Pearson correlation between DNAm GA estimated with the available cord blood (a) and placenta (b) clocks. The regression lines are plotted together with a 95% confidence interval and the Pearson correlation coefficients are depicted. Fig. S6: Scatter plots illustrating the Pearson correlation between estimated (DNAm) and chronological GA for Bohlin's clock (a), Knight's clock (b), Lee's clock (c) and Mayne's clock (d). The regression lines

are plotted together with a 95% confidence interval and the Pearson correlation coefficients are depicted.

Additional file 8. Figure S7: Scatter plots showing the one-tailed Pearson correlation between EAAR estimated in the PREDO cord blood data sets using the beta matrix of median coefficients derived from the final model in ITU and true EAAR values observed in the PREDO cord blood data sets. The regression lines are plotted together with a 95% confidence interval and the Pearson correlation coefficients are depicted.

Additional file 9. Table S3: Median (MED) coefficients, 95% confidence intervals (lcl = lower confidence limit; ucl = upper confidence limit) and % of non-zero of predictor variables across 1000 bootstrap samples for the final elastic net models.

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Authors' contributions
LD analyzed and interpreted the data, prepared and edited tables and figures and wrote and edited the manuscript. ML-P and JL acquired, preprocessed and interpreted data and edited the manuscript. TK contributed to the collection and preprocessing of data. PD contributed to the analysis and interpretation of data and is author of the R package *ensr*. CC was involved in data acquisition and edited the manuscript. HL, SS, PMV, SS-K, JE and EK contributed to the acquisition of data and edited the manuscript. KR and EB contributed to the design of the work, acquired funding and data, interpreted data and revised the manuscript. DC conceptualized the research idea, preprocessed and interpreted data and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
Due to the sensitive nature of the patient data used in the current study and consent, the data sets are not and cannot be made publicly available. However, an interested researcher can obtain a de-identified data set after approval from the PREDO or ITU Study Board. Data requests may be subject to further review by the national register authority and by the ethical committees.

Declarations

Ethics approval and consent to participate
The ITU research protocol has been approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (approval date: 06.01.2015, reference number: 269/13/03/00/09). Each ITU participant has signed a written informed consent form.The PREDO study protocol was approved by the Ethics Committee of Obstetrics and Gynaecology and Women, Children and Psychiatry of the Helsinki and Uusimaa Hospital District and by the participating hospitals. All participants provided written informed consent. Consent of participating children was provided by parent(s)/guardian(s). The study has been registered as ClinicalTrials.gov identifier ISRCTN14030412.

Consent for publication
Not applicable.

Competing interests
EB is the coinventor of FKBP5: a novel target for antidepressant therapy, European Patent no. EP 1687443 B1, and receives a research grant from Böhringer Ingelheim for a collaboration on functional investigations of FKBP5. Otherwise, the authors declare that they have no competing interests.

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Publisher's Note

3. Paper II

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ORIGINAL ARTICLE

Reliability of a novel approach for reference-based cell type estimation in human placental DNA methylation studies

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Abstract

The placenta is a central organ during early development, influencing trajectories of health and disease. DNA methylation (DNAm) studies of human placenta improve our understanding of how its function relates to disease risk. However, DNAm studies can be biased by cell type heterogeneity, so it is essential to control for this in order to reduce confounding and increase precision. Computational cell type deconvolution approaches have proven to be very useful for this purpose. For human placenta, however, an assessment of the performance of these estimation methods is still lacking. Here, we examine the performance of a newly available reference-based cell type estimation approach and compare it to an often-used reference-free cell type estimation approach, namely RefFreeEWAS, in placental genome-wide DNAm samples taken at birth and from chorionic villus biopsies early in pregnancy using three independent studies comprising over 1000 samples. We found both reference-free and reference-based estimated cell type proportions to have predictive value for DNAm, however, reference-based cell type estimation outperformed reference-free estimation for the majority of data sets. Reference-based cell type estimations mirror previous histological knowledge on changes in cell type proportions through gestation. Further, CpGs whose variation in DNAm was largely explained by reference-based estimated cell type proportions were in the proximity of genes that are highly tissue-specific for placenta. This was not the case for reference-free estimated cell type proportions. We provide a list of these CpGs as a resource to help researchers to interpret results of existing studies and improve future DNAm studies of human placenta.

Keywords Cell type estimation · DNA methylation · Human placenta · Chorionic villi · Reference-based deconvolution · Reference-free deconvolution

Introduction

Since the *Developmental Origins of Health and Disease* (DOHaD) hypothesis was proposed, converging evidence supports the high importance of intrauterine conditions for development, as well as for health and disease outcomes later in life [1–3]. The placenta is a complex organ with a central role in fetal development and regulation of the intrauterine environment throughout pregnancy [4–6]. Thus, a better understanding of the placenta's critical role for early development and its molecular landscape is key to disentangling some of the mechanisms driving DOHaD-related developmental aspects [7]. Epigenetic processes are essential for placental development and function, and correspondingly

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healthy fetal development [8, 9]. Consequently, human studies of the placental epigenome are valuable and can help to increase our knowledge about trajectories of health and disease originating in early life.

DNA methylation (DNAm) is one of the most commonly studied epigenetic marks and it is known to be highly tissue- and cell-type-specific. Accordingly, it is important to distinguish direct (true) associations between the exposure of interest and DNAm from associations mediated trough or otherwise caused by placental cell type distributions [10, 11].

To this end, cell type deconvolution algorithms have been developed to retrieve information about cell type composition from DNAm data. They can be mainly categorized into reference-based and reference-free methods [10]. Reference-based cell type deconvolution algorithms rely on biologically defined 5′-C-phosphate-G-3′ (CpG) sites that are uniquely methylated in purified cell types and were identified in a reference sample. For reference-free deconvolution, no a-priori knowledge about differential methylation from purified cell types is necessary, but cell types are predicted directly from DNAm using a computational approach [12]. The first reference-based method to infer changes in the distribution of white blood cells using DNAm signatures was proposed in 2012 by Houseman et al. [13], and pioneering algorithms for reference-free cell type deconvolution were published in 2014 [14, 15]. While reference-free methods are useful when no reference is available, reference-based methods are preferred if a reference is available and there is no evidence for other confounders [10, 16]. To date, the effectiveness of reference-free cell type deconvolution for placenta has not been assessed, and only recently a reference profile for placenta was published [17]. The establishment and validation of this reference in 28 samples constitutes important progress and now allows a reference-based cell type estimation in placenta.

However, an assessment of the performance of this reference-based versus reference-free cell type estimation in placenta with larger study samples is crucial for informing future research. In the current study, we demonstrate the impact of reference-based versus reference-free estimated cell types on DNAm in placental tissue and compare their informativeness. Further, we provide an overview of estimated cell types in placental samples from three independent studies, taken at birth ($n = 470$, $n = 139$, $n = 137$) and, in the largest of these three studies, also during the first trimester ($n = 264$). Our study contributes to a more detailed understanding of human placental characteristics regarding the relatedness of DNAm and cell type composition and underscores the importance of considering cell types in future DNAm studies using placental tissue.

Materials and methods

Study populations

Placental tissue samples were collected from the InTraUterine sampling in early pregnancy (ITU) study, the Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO) study [18], and the Betamethasone (BET) study [19].

ITU and PREDO are Finnish cohort studies consisting of women and their children who were followed throughout pregnancy and beyond. In ITU, women were recruited through the national voluntary prenatal screening program for trisomy 21. If this screening indicated an increased risk of fetal chromosomal abnormalities based on routine serum, ultrasound screening, age and patient history, women were offered further testing including chorionic villus sampling (CVS) at the Helsinki and Uusimaa Hospital District Fetomaternal Medical Center (FMC). During this visit, women were informed about the ITU study. If the chromosomal test indicated no fetal chromosomal abnormality, those who had expressed interest in participating were contacted for final recruitment. Another set of women were informed about ITU when attending the routine screening at maternity clinics. If interest in participating was expressed, they were contacted for final recruitment into the ITU study. In PREDO, the recruitment took place when women attended their first routine ultrasound screening. Some of the women were recruited based on having clinical risk factors for preeclampsia and intrauterine growth restriction, others were recruited independently of these factors [18]. The aim of the BET study was to investigate the effect of antenatal betamethasone on the transplacental cortisol barrier and fetal growth [19]. Pregnant women with preterm labor and cervical shortening were treated with a single course of antenatal BET (Celestan®, MSD GmbH, Haar, Germany) for fetal maturation between 23 + 5 and 34 + 0 weeks of gestation and were recruited prospectively before birth. A gestational-age-matched control group consisted of pregnant women who received no antenatal BET.

Placental tissue samples

In the ITU study, first-trimester placental biopsies were obtained from leftover CVS, following indications of elevated risk for chromosomal abnormalities between 10 and 15 weeks of gestation. Placenta samples were also collected at birth, whereby midwives/trained staff took nine-site biopsies (within maximum 120 min after delivery) from the fetal side of the placenta, at 2–3 cm from umbilical cord insertion. In the PREDO study, placenta nine-site biopsies

(within maximum 90 min after delivery) were taken from the decidual side of the placenta. In the BET study, full-thickness placental biopsies were taken by a uniform random sampling protocol [20, 21] from both peripheral and central areas. All samples were stored at − 80 °C.

Throughout the manuscript, we refer to all placental samples collected at birth as 'term placenta', and to all placental CVS samples collected during early pregnancy as 'CVS'.

DNA methylation (DNAm)

From the collected samples, DNA was extracted according to standard procedures and DNAm was assessed using the Illumina Infinium MethylationEPIC array (Illumina, San Diego, USA). In total, DNA methylation levels were assessed in 1055 samples: $n = 277$ CVS samples (ITU), and $n = 500$ placental samples (ITU), $n = 140$ placental samples (PREDO), and $n = 138$ placental samples (BET) taken at birth. All DNAm data were pre-processed in the same way, using an adapted pipeline from Maksímovíc et al. [22] and the R package *minfi* [23]. Beta values were normalized using stratified quantile normalization [24], followed by BMIQ [25]. Batch-effects were removed using *ComBat* [26].

The final data sets comprised 264 CVS samples from ITU ($n = 716{,}331$ probes) and 486 placental samples ($n = 665{,}190$ probes) from ITU, 139 placenta samples ($n = 755{,}154$ probes) from PREDO and 137 placenta samples ($n = 708{,}222$ probes) from the BET study. Of these, 652,341 probes overlapped across all four data sets.

Gestational age, child sex and ethnicity variables

Gestational age (GA) at sampling was based on fetal ultrasound. Child sex was extracted from the Finnish Medical Birth Register (MBR) in ITU and PREDO and obtained from postnatal assessment in the BET study. To retrieve information about genetic background, we performed multi-dimensional scaling (MDS) analysis on the identity-by-state (IBS) matrix of quality-controlled genotypes [27]. We used the first two components for ITU and PREDO and the first four components for the BET study, as it was ethnically more heterogenous. In the following, we refer to these MDS components as 'PC 1/2/3/4 ethnicity', respectively. This information was available for $n = 200$ individuals with CVS tissue in ITU, and $n = 439$ individuals with term placental tissue in ITU, in $n = 118$ individuals with term placental tissue in PREDO and $n = 136$ individuals with term placental tissue in BET. Genotyping was performed on Illumina Infinium Global Screening arrays for BET and ITU and on Illumina Human Omni Express Arrays for PREDO. DNA for genotyping was extracted from cord blood in ITU and PREDO, if available, otherwise placental tissue was used in ITU. DNA was extracted from placental tissue in the BET study. Further details about genotypic assessment and quality control

Table 1 Study sample characteristics [*Mean* (*SD*) or *N* (%) for each variable]

	ITU		PREDO	BET
	CVS	Placenta	Placenta	Placenta
Sample size	264	470	139	137
Phenotypes				
Gestational age	12.79 (0.82)	39.99 (1.55)	39.89 (1.43)	38.16 (1.95)
Child sex (male)	140 (53%)	238 (51%)	67 (48%)	70 (51%)
Reference-based cell types				
Trophoblasts	0.26 (0.06)	0.01 (0.03)	0.04 (0.05)	0.13 (0.06)
Stromal	0.17 (0.06)	0.01 (0.02)	0.04 (0.03)	0.11 (0.02)
Hofbauer	0.00 (0.01)	0.00 (0.01)	0.00 (0.00)	0.00 (0.00)
Endothelial	0.00 (0.01)	0.01 (0.02)	0.08 (0.03)	0.11 (0.02)
nRBC	0.00 (0.01)	0.04 (0.03)	0.00 (0.01)	0.00 (0.00)
Syncytiotrophoblasts	0.57 (0.04)	0.93 (0.06)	0.83 (0.08)	0.66 (0.08)
Reference-free cell types				
C1	0.26 (0.14)	0.11 (0.09)	0.43 (0.19)	0.35 (0.2)
C2	0.30 (0.15)	0.07 (0.07)	0.51 (0.20)	0.46 (0.2)
C3	0.14 (0.07)	0.23 (0.13)	–	0.14 (0.1)
C4	0.10 (0.07)	0.13 (0.09)	–	–
C5	0.14 (0.10)	0.13 (0.09)	–	–
C6	–	0.11 (0.08)	–	–
C7	–	0.09 (0.07)	–	–
C8	–	0.08 (0.07)	–	–

in the ITU and PREDO cohorts, as well as in the BET study, have been published elsewhere [28, 29].

An overview of study sample characteristics is given in Table 1.

Cell type composition estimation

Reference-based cell type composition into six cell types (nucleated red blood cells, trophoblasts, syncytiotrophoblasts, stromal, Hofbauer, endothelial) was estimated using a reference recently published by Yuan et al. [17] and implemented within the R package *planet*, by applying the robust partial correlation algorithm [30].

The result of this cell type estimation is the amount of the respective cell types in every person, while all estimated cell types add up to 100%.

Reference-free cell types were estimated following the protocol suggested in the R package *RefFreeEWAS* [31], which led to five estimated 'cell types' in CVS (ITU), and eight estimated 'cell types' (ITU), two estimated 'cell types' (PREDO) and three estimated 'cell types' (BET) in term placenta. We refer to cell types here, although the output of this procedure does not give explicit cell types, but latent quantities and their respective proportion for every person.

Statistical analyses

All statistical analyses were performed in R, version 4.0.5/4.1.1 [32].

Filtering of invariable probes in DNAm

To assess the influence of cell types on DNAm, we first filtered for variable CpGs by excluding placenta-specific non-variable CpGs. We applied a procedure described by Edgar et al. [33] to the overlapping CpGs ($n = 652{,}341$) of all four placental methylation data sets from the EPIC array, to identify sites with < 5% range between 10 and 90th percentile in DNAm beta values using our data sets. This resulted in 120,548 CpGs (listed in Supplementary Table S1) that we identified as non-variable for placental EPIC methylation data and excluded from further analyses. Identifying these CpGs is useful to reduce dimensionality, and becomes especially relevant for future studies, e.g., epigenome-wide association studies (EWAS), aiming to use our resources. Furthermore, the 1050 CpGs used to predict cell type composition in the model by Yuan et al. [17] were excluded from the following analyses to prevent circular conclusions.

Capturing DNAm variance through principal components and filtering of individuals

To capture the major variance in DNAm, we performed singular value decomposition on methylation beta values, and extracted the first principal component (PC1) explaining most of the variance for every data set (Supplementary Fig. S1). For term placenta from ITU we identified $n = 16$ outliers representing values greater than three times interquartile-range in PC1 (see Supplementary Fig. S2a). The same samples showed lower sample-sample correlations in DNAm beta values with the other placenta samples (Supplementary Fig. S2b) and presented different cell type proportions (Supplementary Fig. S2c). Thus, we excluded these samples from the ITU placenta data set, resulting in $n = 470$ term placenta samples from the ITU cohort. We calculated the principal components (PC) without these outliers in the ITU term placenta data set. For CVS from ITU and term placenta data sets from PREDO and BET no such outliers were identified.

Correlation of reference-free estimated cell types with reference-based estimated cell types and phenotypes

Spearman's rank correlations were calculated both between reference-free and reference-based estimated cell types and between reference-free estimated cell types and phenotypes (GA, child sex, ethnicity PCs and additionally fetal chromosomal testing and BET administration status in the ITU and BET placenta, respectively) in every tissue. Adjustment for multiple testing was done using Bonferroni correction.

Models to predict DNAm by cell type proportions (reference-based versus reference-free)

To compare the impact of reference-based versus reference-free estimated cell types on the main variance in DNAm, PC1 of DNAm beta values was regressed linearly on different predictors in six models for every data set:

1. PC methylation ~ 1
2. PC methylation ~ GA at sampling + child sex + PCs ethnicity
3. PC methylation ~ reference-based estimated cell types
4. PC methylation ~ reference-based estimated cell types + GA at sampling + child sex + PCs ethnicity
5. PC methylation ~ reference-free estimated cell types
6. PC methylation ~ reference-free estimated cell types + GA at sampling + child sex + PCs ethnicity

Using cross-validation with 10 folds, 500 repeats and *RMSE* as loss function, implemented in the R package

xvalglms [34], enabled us to evaluate which model best explains variability in placental DNAm. This is defined by the number of times a particular model wins in the repeated cross-validation procedure, i.e., the number of times that the model has a smaller prediction error (*RMSE*, in our case) than all other models considered. *RMSE* is on the same scale as the outcome variable and the partitions of data were the same for all models. As *RMSE* is not comparable between the data sets, we additionally report the adjusted R^2 values of the winning models.

For the BET data set, we observed outliers in *RMSE* in some of the repeats (see Supplementary Fig. S3a). After further exploration it became evident that these were driven by five samples, which were different in Hofbauer and nRBC cell type proportions, i.e., all samples apart from these five had no estimated proportions of Hofbauer and nRBC cells (see Supplementary Fig. S3b). We also tested if outliers in any of the other estimated cell types (see Supplementary Fig. S3c) changed the behavior of the model, but this was not the case. Furthermore, outliers were present in all data sets and are not suspicious per se in samples from heterogenous tissue like placenta. Thus, we only excluded the five samples presenting very different in estimated Hofbauer and nRBC cells in the BET data set from this analysis.

We further tested how much of DNAm variability in all single CpGs could be explained by either reference-based or reference-free estimated cell types. Linear models were fitted for every CpG by predicting DNAm (beta values) with either reference-based or reference-free cell types. For every CpG, the adjusted R^2 was extracted (see Supplementary Fig. S4 for a histogram of R^2 values). Afterwards, CpGs with adjusted $R^2 > 0.30$ in all four data sets were extracted and considered as CpGs at which variability of DNAm (beta values) was relatively strongly influenced by cell type proportions. We decided to use this criterion based on an evaluation of the histograms (Fig. S4) and as the mean adjusted R^2 values of the 90% quantile of all data sets was $R^2_{\text{Adjusted}} = 0.30$, and our aim to only extract the most informative CpGs, i.e., to be rather strict in this selection. For the following enrichment analyses, the genes (20,038) mapping to all CpGs (534,510) overlapping between the data sets were used as background.

Enrichment analyses

All CpGs were mapped to the closest gene using the R package *bumphunter* functions *annotateTranscripts* and *matchGenes* [35]. Afterwards, the genes corresponding to the extracted CpGs were used as input for the *TissueEnrich* package [36], while the genes corresponding to all CpGs overlapping between the data sets (without any filtering for R^2) were considered as background genes ($n = 20{,}038$). The same input and background genes were further used for the *PlacentaCellEnrich* Tool [37]. Human placental single-cell RNA-Sequencing data [38] were used to retrieve enrichments for placenta cell-specific expression patterns. For both enrichment analyses we used an adjusted p value of 0.01 as threshold for enrichment, as recommended by the authors of the *PlacentaCellEnrich* Tool [37].

Cell type composition analyses

Differences in reference-based cell type proportions between the three term placenta data sets were analyzed using non-parametric global multivariate analysis of variance [39] implemented in the R package *npmv* [40]. To test for significant differences between the study groups, we applied the global test using the *R* function *nonpartest* with default settings, which provides *F*-distribution approximations, performs multivariate permutation and calculates nonparametric relative effects. The global test was supplemented with a more detailed comparison (*R* function *ssnonpartest*) of study groups and cell types using the *F* approximation of Wilks' lambda, to identify which variables/factor levels contribute to the significant differences, while controlling for the familywise error rate ($\alpha = 0.01$).

Differences in reference-based cell type proportions between CVS and term placenta from the same individuals ($n = 85$, ITU) were calculated using paired Wilcoxon signed-rank tests. All p values were corrected for multiple testing ($n = 6$ cell types) using Bonferroni correction and compared to $\alpha = 0.01$.

Spearman correlations and Wilcoxon signed-rank tests were performed to test for relationships between reference-based cell type proportions and GA and child sex (for every cell type separately and corrected for multiple testing among the $n = 6$ cell types using Bonferroni correction and $\alpha = 0.01$).

Results

Reference-free estimated cell types do not map to reference-based estimated cell types and are correlated with child sex

For an illustration of the correspondence between reference-based and reference-free estimated cell types, Spearman correlation coefficients are shown in Fig. 1. Although there were some correlations between reference-based and reference-free estimated cell types, there was no clear matching between reference-based estimated cell types and specific reference-free components. Furthermore, Spearman correlation coefficients for reference-free estimated cell types and included phenotypes are depicted in Fig. 2. It can be seen that especially child sex was correlated with the reference-free estimated cell type components.

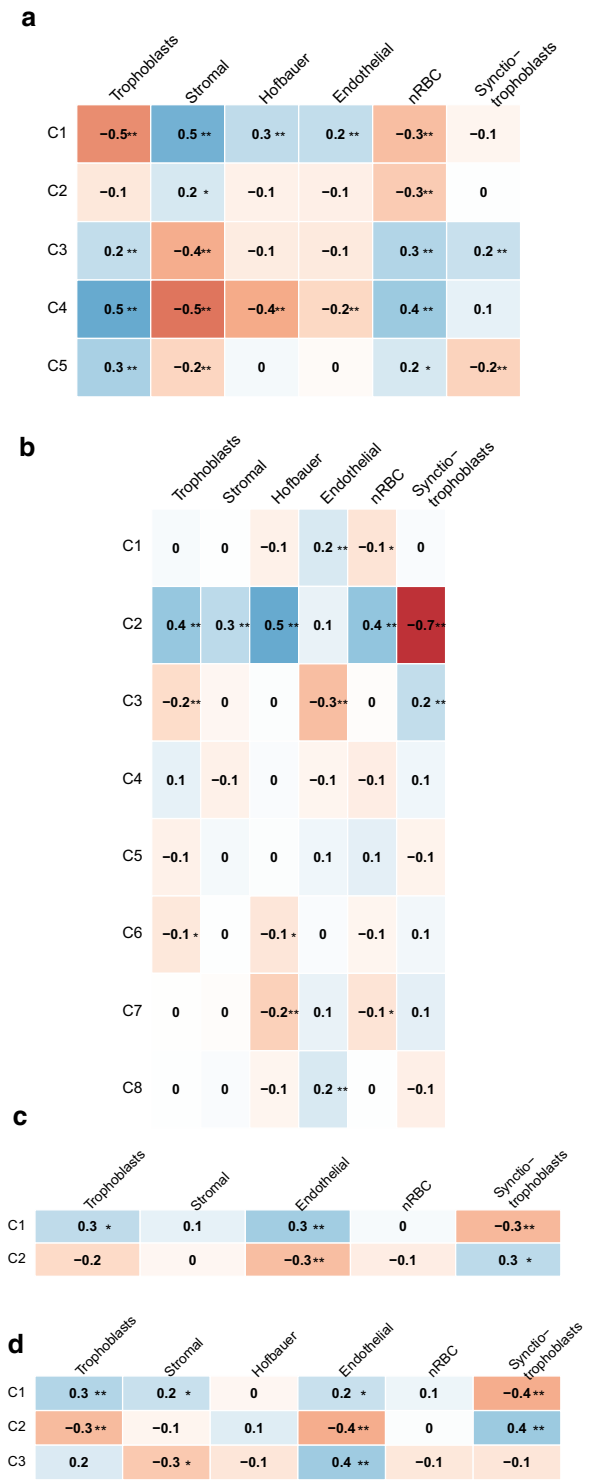

Fig. 1 Plot of the Spearman correlation coefficients (**$p < 0.001$, *$p < 0.01$) between reference-based and reference free estimated cell types in **a** first trimester placenta (CVS) from ITU, **b** term placenta form ITU, **c** term placenta from PREDO and **d** term placenta from the BET study

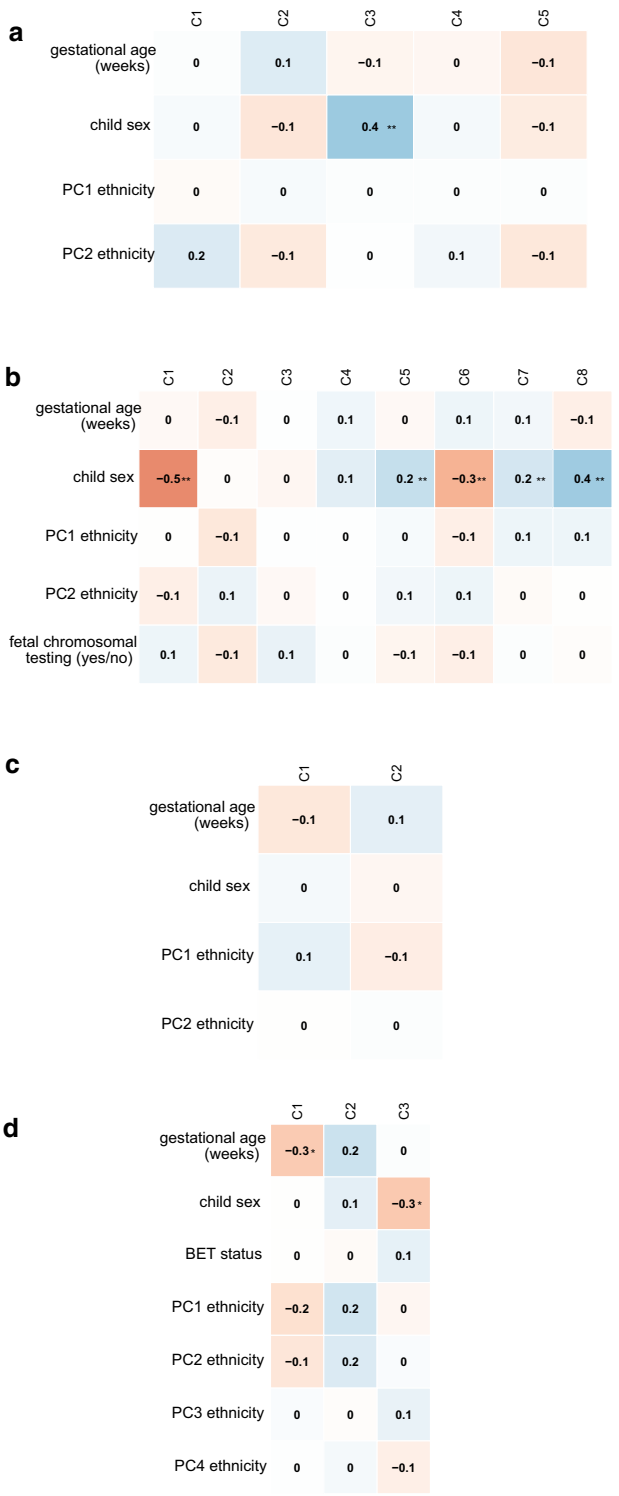

Fig. 2 Plot of the Spearman correlation coefficients (**$p < 0.001$, *$p < 0.01$) between reference free estimated cell types and phenotypes in **a** first trimester placenta (CVS) from ITU, **b** term placenta form ITU, **c** term placenta from PREDO and **d** term placenta from the BET study

For the majority of data sets, reference-based methods predict variability of DNAm better than reference-free methods

To evaluate the impact of phenotypic variables (GA, child sex, ethnicity) vs. reference-based vs. reference-free cell type composition on the main variance in DNAm (PC1), we compared the predictive performance of six competing models: an intercept-only model (model 1), phenotype model (model 2), reference-based cell type model with or without phenotypes (model 3 and 4) and reference-free cell type model with or without phenotypes (model 5 and 6). All models were tested in each data set among individuals with complete information available ($n = 200$ for CVS from ITU, $n = 425$ for term placenta from ITU, $n = 118$ for term placenta from PREDO and $n = 136$ for term placenta from the BET study with five outliers excluded (see "Materials and methods") resulting in $n = 131$).

The results of the cross-validation procedure for model selection are shown in Fig. 3. Models including cell type estimations always performed better than the intercept-only model (model 1) or a model including only phenotypes (GA, sex, ethnicity; model 2). In CVS data (Fig. 3a), the model including reference-based cell types only (model 3) gave the most accurate out-of-sample predictions of PC1 (80% of the wins), with an average prediction error of 79.58 (95% CI [78.57, 80.89]), followed by the model including reference-based cell types and phenotypes. The adjusted R^2 of the winning model was $R^2_{\text{Adjusted}} = 0.90$.

Placental samples taken from the fetal side at birth in the ITU cohort were the only data set where reference-free cell types outperformed reference-based cell types in the prediction of PC1 DNAm (Fig. 3b). In this data set, the model including both reference-free cell types and phenotypes (model 6) always won, presenting with an average prediction error of 72.62 (95% CI [71.97, 73.34]). The adjusted R^2 of the winning model was $R^2_{\text{Adjusted}} = 0.92$. These results did not change when information about fetal chromosomal testing (yes or no) was included as an additional phenotype variable in the models. In PREDO (Fig. 3c), where the placental samples were taken from the decidual side at birth, the model including reference-based cell types together with phenotypes (model 4) performed best (79% of wins) with an average prediction error of 111.44 (95% CI [107.08, 121.70]. In the BET study (Fig. 3d), where placental biopsies spanning from the decidual to the fetal side were collected at birth, the model including reference-based cell types (model 3) won in most of the repeats (99% of wins) with an average prediction error of 87.84 (95% CI [86.48, 89.54]. When including BET (administered or not) as a phenotype variable for the BET study, the winning model was still model was still the model including only reference-based estimated cell types (model 3). The adjusted R^2 of the winning model was $R^2_{\text{Adjusted}} = 0.86$ in both the PREDO and BET placenta.

In both PREDO and BET, the second-best model was the other model including either both reference-based estimated cell types and phenotypes (model 4, for BET) or only reference-based cell types (model 3, for PREDO).

The conclusions from predicting DNAm variability in single CpGs by either reference-based or reference-free estimated cell types were concordant with the model for PC1 in DNAm. On average, reference-based cell types explained more variance (adjusted R^2) in DNAm compared to reference-free cell types among CpGs in CVS from ITU ($n = 264$; R^2_{Adjusted} $M = 0.13$, $SD = 0.17$ vs. $M = 0.12$, $SD = 0.12$), and in placental tissues at birth in PREDO ($n = 139$; R^2_{Adjusted} $M = 0.11$, $SD = 0.16$ vs. $M = 0.05$, $SD = 0.06$), and in BET ($n = 137$; R^2_{Adjusted} $M = 0.10$, $SD = 0.13$ vs. $M = 0.06$, $SD = 0.07$). Only placental tissues sampled at birth in ITU ($n = 470$), reference-free estimated cell types explained more of the variance in DNAm (R^2_{Adjusted} $M = 0.18$, $SD = 0.18$) than reference-based estimated cell types (R^2_{Adjusted} $M = 0.11$, $SD = 0.15$).

CpGs with larger proportions of variability explained by reference-based cell types map to placenta-specific genes

CpGs where estimated cell type composition explained more than 30% of variance (adjusted $R^2 > 0.3$) in all four data sets were considered as CpGs at which variability was relatively strongly influenced by cell type proportions. A list of these CpGs and corresponding genes can be found in Supplementary Table S2. For the reference-based model, this was the case for 26,092 CpGs mapping to 8511 genes. For the reference-free model, this was true for 531 CpGs mapping to 398 genes.

The results of the tissue enrichment analyses can be seen in Fig. 4. When using the reference-based estimated cell types, genes mapping to CpGs where variability was strongly influenced by cell types were enriched for placenta-specific genes (Fig. 4a, $p < 0.001$ and fold-change = 1.291. We provide a list of these 186 placenta-specific genes in Supplementary Table S3. For reference-free estimated cell types, genes mapping to CpGs where variability is strongly influenced by cell types were not enriched for placenta-specific genes (Fig. 4b): only 10 genes were found to be placenta-specific. However, there was an enrichment for cerebral cortex, with $p < 0.001$, fold-change = 2.209.

Next, we ran cell-specific enrichment analysis using a placenta-specific dataset (*PlacentaCellEnrich* Tool). Cell-specific expression patterns can be seen in Fig. 5. Again, the results reflect a higher placenta-specificity when using the reference-based approach (Fig. 5a), showing a significant enrichment for a number of placental cells as follows: syncytiotrophoblasts, villous cytotrophoblast, extravillous

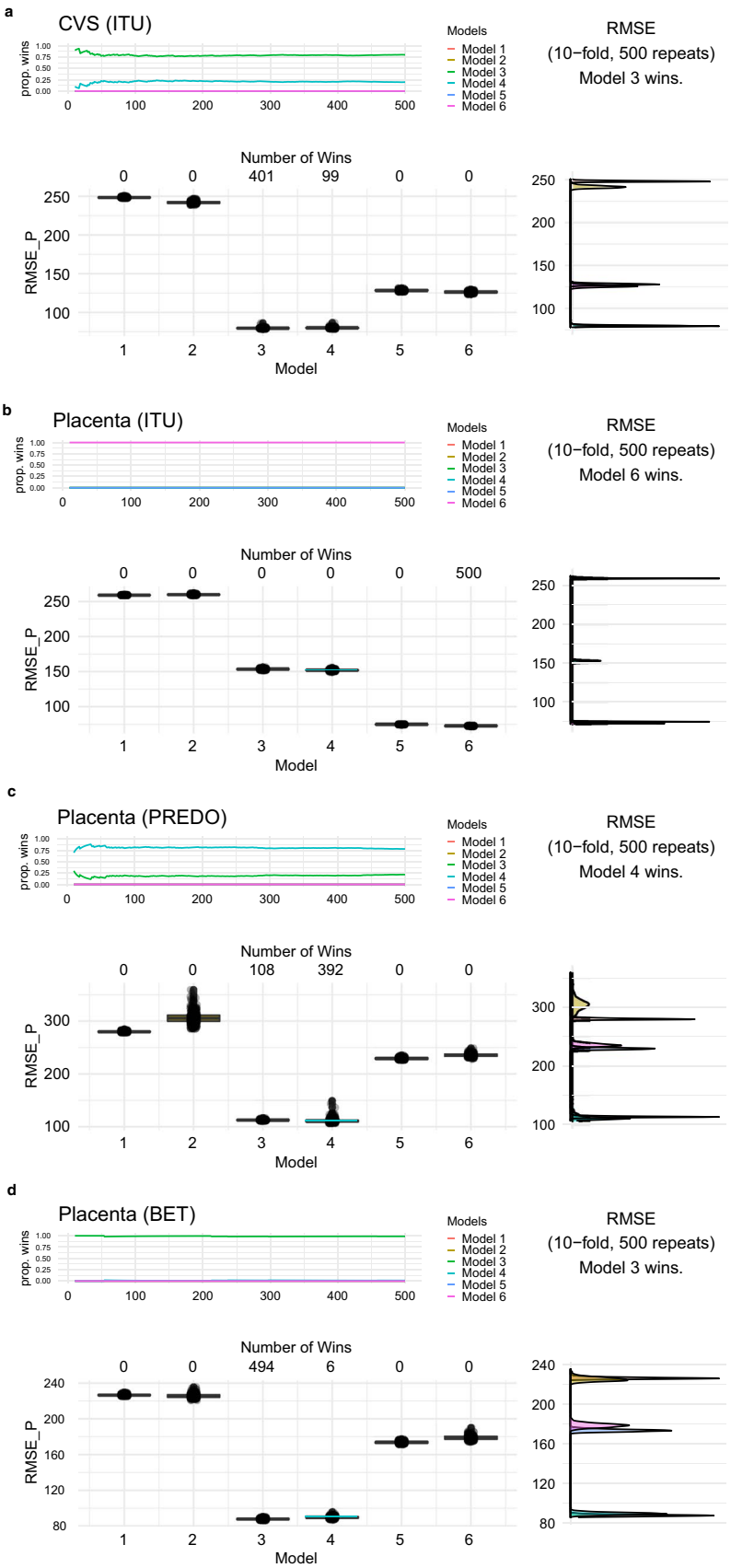

Fig. 3 Cross-validation results for predicting PC1 of DNAm comparing 6 models (model 1 = intercept-only; model 2 = phenotypes (gestational age (GA), child sex, ethnicity); model 3 = reference-based estimated cell types; model 4 = reference-based estimated cell types and phenotypes; model 5 = reference-free estimated cell types; model 6 = reference-free estimated cell types and phenotypes). The upper panel illustrates the proportions of wins among all repetitions for each model (models with zero wins overlap and hence not all colors are displayed), and the winning model is listed. The panel below shows the boxplots of the prediction error (root mean square error of prediction, $RMSE_p$) for all six models with the number of wins for each model displayed at the top. The panel on the right is a graph of density estimates for the prediction errors. Models were compared independently in four different tissue samples, **a** first trimester placenta (CVS) from ITU, **b** term placenta form ITU, **c** term placenta from PREDO and **d** term placenta from the BET study

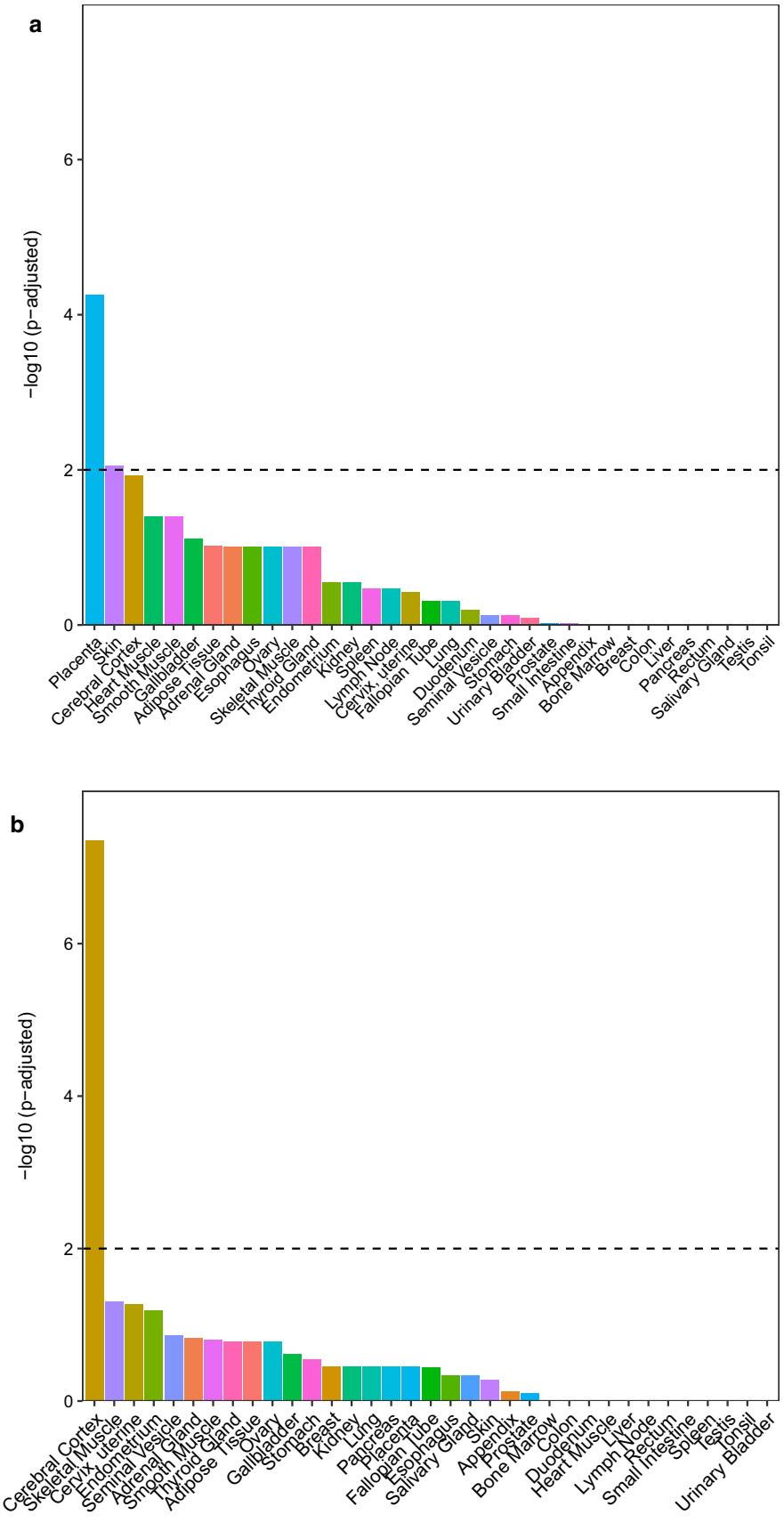

Fig. 4 Tissue enrichment among genes mapped to CpGs with a minimum of 30% explained variance in DNAm predicted by cell type proportions from **a** reference-based cell type estimation and **b** reference-free cell type estimation

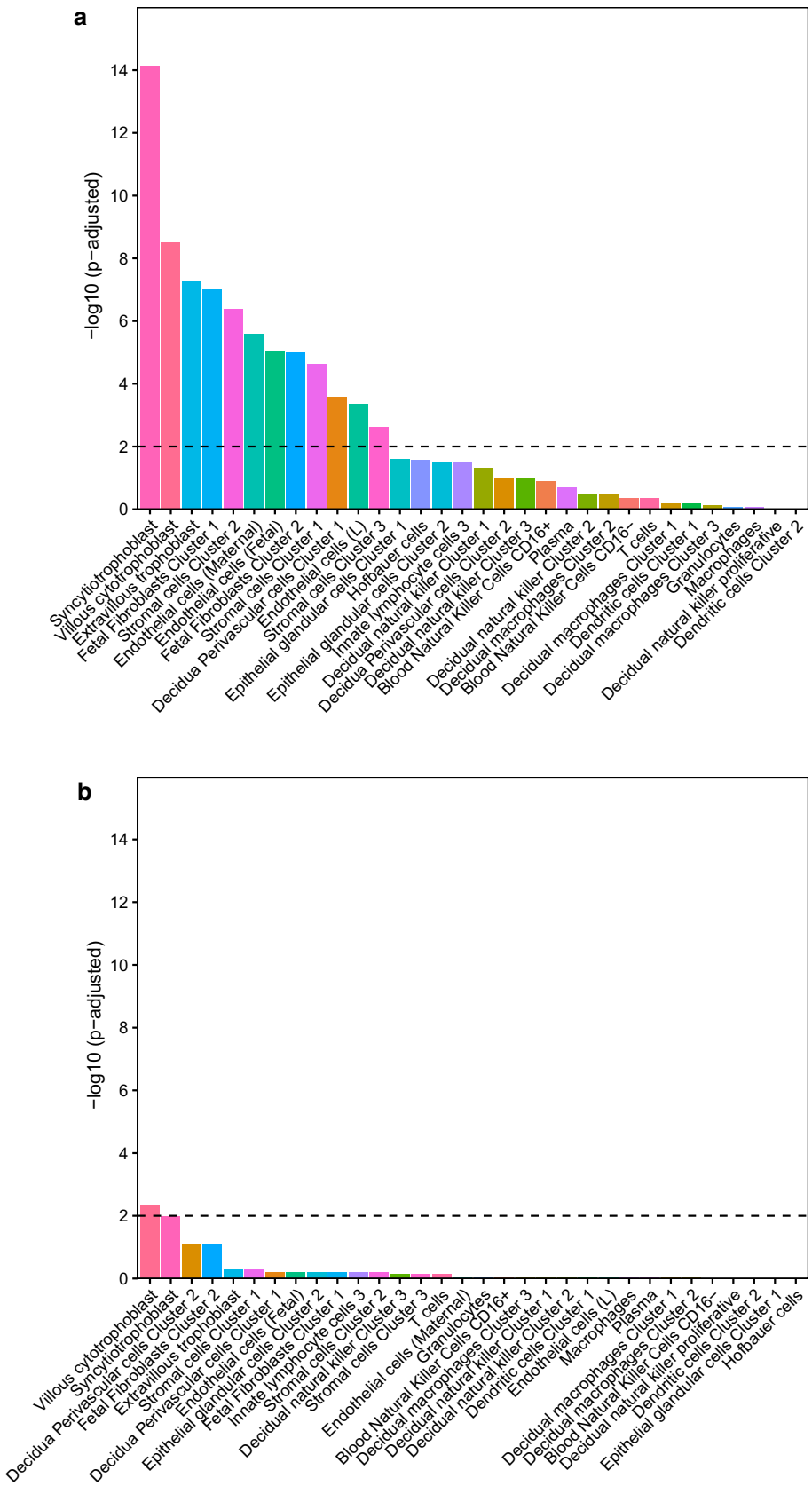

Fig. 5 Enrichment for placental cell-specific genes among genes mapped to CpGs with a minimum of 30% explained variance in DNAm predicted by cell type proportions from **a** reference-based cell type estimation and **b** reference-free cell type estimation

trophoblast, fetal fibroblasts, stromal cells, endothelial cells and decidua perivascular cells. These represent the major cell types in the placenta [41], indicating that this approach accounted for the majority of confounding possible from cell type heterogeneity. Using the reference-free approach (Fig. 5b) there was only an enrichment of villous cytotrophoblasts. A summary of parameters of the cell-specific enrichment can be found in Supplementary Table S4.

Cell type composition

We next wanted to estimate the cell type proportions in the different study samples using the reference-based method (Fig. 6).

Cell type proportions in term placentas show differences between studies

While cell type estimates were highly similar for samples within a study, we observed significantly different estimated cell type proportions among the three studies with placental samples collected at birth, according to each of the four test criteria (ANOVA type, Lawley-Hotelling type, Bartlett–Nanda–Pillai type, and Wilks' lambda type). Test statistics are given in Supplementary Table S5. Nonparametric relative effects, quantifying the probability that a value obtained from one study sample is larger than a value randomly chosen from the other study samples, are provided in Supplementary Table S6. The post-hoc testing procedure following the global test determined that samples from all three studies and all cell types contributed to these significant differences. In all three term placenta data sets, syncytiotrophoblasts were the main estimated cell type, but the highest proportion was estimated in term placenta from ITU. Estimates for proportions of trophoblasts, stromal and endothelial cells were highest in the BET study sample, followed by term placenta from PREDO.

Cell type proportions show intra-individual changes from CVS to term Placenta

The estimated cell type proportions differed significantly between early-pregnancy CVS and placenta sampled at birth for a number of cell types. Largest differences in estimates were observed for stromal cells ($Mdn = 17.4\%$ in CVS vs. $Mdn = 0.0\%$ at birth, $Z = 8.0$, $p < 0.001$), syncytiotrophoblasts ($Mdn = 56.9\%$ in CVS vs. $Mdn = 95.3\%$ at birth, $Z = -8.0$, $p < 0.001$), and trophoblasts ($Mdn = 24.8\%$ in CVS vs. Mdn = 0.0% at birth, $Z = 8.0$, $p < 0.001$) followed by endothelial cells ($Mdn = 0.0\%$ in CVS vs. $Mdn = 0.4\%$ at birth, $Z = -6.1$, $p < 0.001$), nRBC ($Mdn = 0.0\%$ in CVS vs. $Mdn = 3.2\%$ at birth, $Z = -7.7$, $p < 0.001$). This was based on 85 individuals from the ITU cohort for whom both CVS and placenta tissue at birth were available. Syncytiotrophoblasts were the most abundant estimated cell type in both CVS and term placenta tissue, but there was a strong median increase of 38.4% in this cell type from early-pregnancy to birth. The largest decrease from early-pregnancy to birth was in estimated trophoblasts from CVS to term placenta (median decrease of 24.8%), followed by estimated stromal cells (median decrease of 17.4%).

Associations between reference-based estimated cell types and gestational age

Finally, we wanted to see whether the estimated cell type proportions follow physiological changes over gestation.

Higher GA at sampling was significantly related to lower estimated trophoblast proportions in CVS ($r_s = -0.32$, $p < 0.001$) and term placenta from the BET study ($r_s = -0.42$, $p < 0.001$), and to higher estimated syncytiotrophoblast proportions in CVS ($r_s = 0.36$, $p < 0.001$) and term placenta from the BET study ($r_s = 0.37$, $p < 0.001$). The effects were not significant, though in the same direction, for the other two data sets (term placenta from ITU and PREDO), where GA was more skewed towards higher gestational age. The relationship of estimated trophoblast and syncytiotrophoblast proportions with GA is shown in Fig. 7.

We observed no significant relationships with GA among the other estimated cell types.

Similar to Yuan et al. [17] we observed no significant sex-specific differences in estimated cell type proportions in any of the study samples.

Discussion

In this study, we examined a new DNAm-based reference which enables reference-based cell type estimation in placenta [17] in a large data set comprising over 1000 samples from three independent studies, with $n = 746$ placental samples collected at birth, and $n = 264$ during the first trimester of pregnancy. We investigated intra- as well as inter-individual differences in estimated cell type proportions. Furthermore, we compared the reference-based to a reference-free approach (namely, RefFreeEWAS) [31], regarding its potential to control for cell type proportions in DNAm studies of human placenta. We provide lists of CpGs from the EPIC

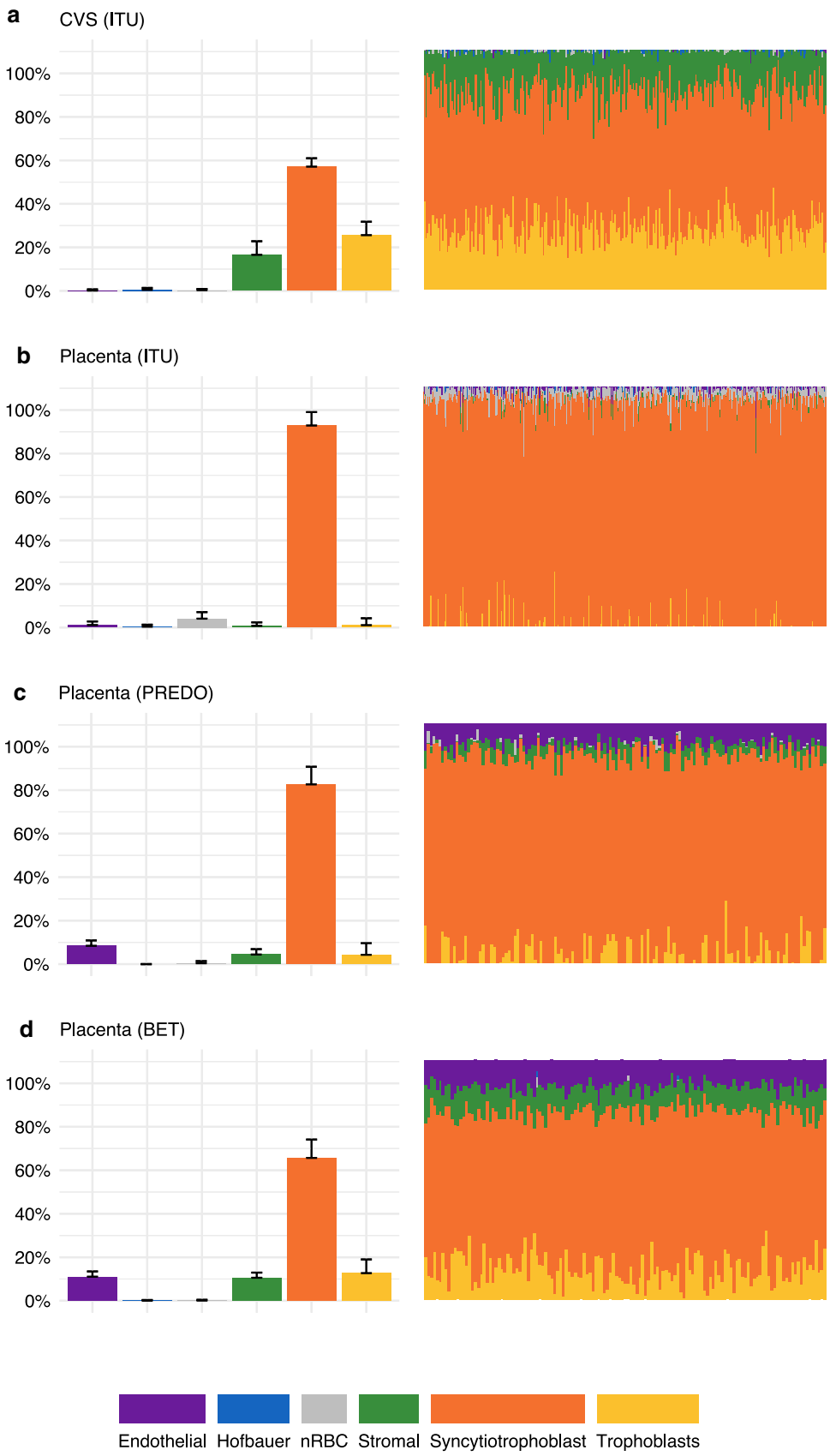

Fig. 6 Depicted are the mean and standard deviation of the reference-based estimated cell type's proportion (raw estimates using the reference by Yuan et al. [17] and robust partial correlation algorithm) together with an illustration of the relative estimated cell type proportion in **a** $n=264$ individuals in CVS from ITU, **b** $n=470$ individuals in term placenta from ITU, **c** $n=139$ individuals in term placenta from PREDO and **d** $n=137$ individuals in term placenta from the BET study

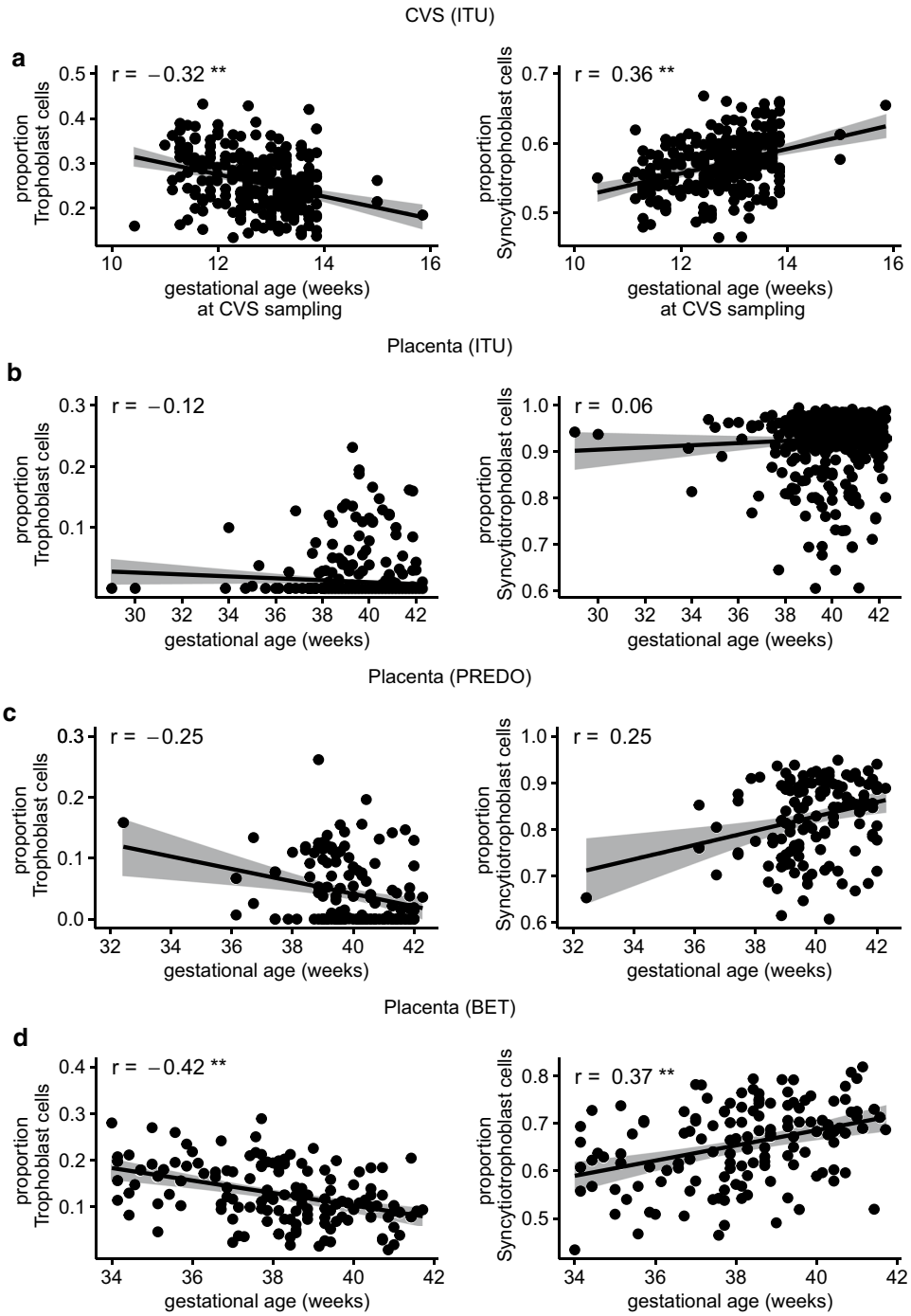

Fig. 7 Scatterplots showing the Spearman correlation (**p value < 0.001) of trophoblast and syncytiotrophoblast proportions with gestational age in **a** first trimester placenta (CVS) from ITU ($n = 264$), **b** term placenta form ITU ($n = 470$), **c** term placenta from PREDO ($n = 139$) and **d** term placenta from the BET study ($n = 137$)

array which we found to be (1) non-variable in placental tissue (Supplementary Table S1), and (2) highly influenced by cell types (Supplementary Table S2).

Using a cross-validation model focusing on the prediction of the major variance in DNAm, as well as an investigation at single CpGs level, we confirmed the importance of cell type composition for variability in DNAm.

At the same time, the latter shows that it is a select subset of CpGs where the impact of cell type proportions on DNAm is especially important (Supplementary Table S2).

Both reference-free and reference-based cell type estimation methods can account for variability in DNAm. However, for the majority of data sets, the reference-based approach better predicted variability of DNAm.

Generally, reference-based cell type estimation allows for a more direct interpretation of cell type composition. This

was underscored by the fact that the overlap in CpGs with high amount of DNAm variability explained by estimated placental cell types was much more consistent among the different data sets when using reference-based cell types (26,092 CpGs) versus reference-free cell types (531 CpGs). Furthermore, genes mapping to these CpGs with high proportions of DNAm variability explained by estimated reference-based cell types were enriched for placenta-specific genes, while this was not the case when using the reference-free approach (see Fig. 4). A possible reason for this could be that the reference-free methods do not only depict cell types, but further unknown sources of variance, and as such it is difficult to interpret what the estimated reference-free 'cell types' actually reflect. This also becomes clear from Figs. 1 and 2, where we depict that reference-based estimated cell types are not highly correlated with a specific reference-free cell type component, but rather with child sex. This might also explain why in one of the term placenta data sets DNAm variability was better explained by reference-free compared to reference-based estimated cell types - probably not only cell types were covered by the estimated 'cell types' which contributed to DNAm variability in the complex tissue samples. This could suggest that even though reference-based cell type correction approaches outperform reference-free approaches in most settings, cohort-specific differences may affect the performance of these methods.

Overall, considering the performance of the reference-based cell type estimation, it may be advisable to use reference-based methods, such as from Yuan et al. [17] in future studies investigating DNAm in human placenta.

Higher GA was associated with higher proportions of syncytiotrophoblasts and lower proportions of trophoblasts in the placenta samples collected at birth (Fig. 7). This finding was congruent with the changes in estimated cell type composition we observed from first trimester to birth placenta samples from the same individuals: trophoblast cells showed the largest decrease, syncytiotrophoblasts the largest increase. These differences in the estimated cell type proportions between early and late pregnancy are probably reflective of placental maturation process [42]. Trophoblasts give rise to further subpopulation of cells and syncytiotrophoblasts expand during pregnancy [5]. Yuan et al. [17] reported an increase in estimated syncytiotrophoblasts and endothelial cells and decrease in stromal cells from first trimester to term placenta samples, which is again concordant with our results despite their comparison of samples from different individuals, in contrast to our within-sample design in 85 individuals. Nevertheless, it should be mentioned that we cannot rule out that some of the differences in estimated cell type proportions may arise from differences in sampling and storage conditions of the CVS and the placental tissue.

Regarding child sex, Yuan et al. did not find any association with estimated cell composition [17]. We can confirm this result, as there was no evidence for sex-specific differences in reference-based estimated cell type composition.

Additionally, the use of three independent studies (ITU, PREDO, BET) enabled us to investigate between-study differences in estimated cell type proportions at birth. We observed that cell type composition was rather consistent among samples within a study but different between studies. The larger variance in cell type proportions between studies (versus between individuals within a study) might reflect the different sampling schemes of placental tissue (see "Materials and methods"). The placenta is a highly complex organ, which makes the sampling procedure difficult and particularly prone to differences between studies [21, 43].

An important strength of our study is that we were able to investigate placental cell type composition in a large number of placentas from different independent studies. In addition to examining placental DNAm at birth, we included early-pregnancy placental CVS samples: in a subset of 85 individuals, longitudinal data on placental DNAm both in early pregnancy and at birth were available, giving us the rare chance to examine change over time within the same placentas. We also provide resources that can be used for the interpretation and design of DNAm studies in placenta, especially EWAS. However, there are also some limitations: we rely on bioinformatic indirect deconvolution, which also limits our investigation to the cell types included in the reference sample [17]. This was in turn limited by the availability of unique markers suitable for cell type selection using fluorescence-activated cell sorting, and dissection accuracy. Future tools based on single-nucleus DNA methylation analyses would undoubtedly improve cell type accuracy as well as diversity, thus improving usefulness for deconvolution in bulk tissue analyses. Furthermore, we only compared one reference-based deconvolution to one of several (semi-) reference-free approaches available [16]. Thus, our comparison of performance between methods is limited to these chosen approaches and is only an indication of the ability of the reference-based method to account for variability in DNAm compared to another often-used reference-free approach, but not generalizable to all reference-free methods. Additionally, we only used the first principal component of DNAm in the cross-validation procedure for model comparison, which is a reduction of dimensionality and improves interpretability, but at the same time can only capture part of the total variation in the data.

Overall, addressing cell type heterogeneity in studies of DNAm is important to avoid misinterpretation of results, to limit confounding and increase precision by distinguishing changes in cell type proportions from epigenetic changes due to other factors, such as for example environmental

exposures [44]. Apart from this, cell type composition is also an important factor to consider for understanding gene regulatory mechanisms in human tissues [45] and tissue function overall. This study contributes to a more detailed understanding of the interrelation between DNAm and estimated cell type composition in human placenta and stands as a resource to help researchers design future DNAm studies of human placenta and interpret results of both existing and future studies.

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Availability of data and materials Due to the sensitive nature of the patient data used in the current study and consent, the data sets are not and cannot be made publicly available for data protection reasons.

Code availability R Code for the main analyses is provided here: https://doi.org/10.5281/zenodo.5713621.

Declarations

Conflict of interest EB is the coinventor of FKBP5: a novel target for antidepressant therapy, European Patent no. EP 1687443 B1, and receives a research grant from Böhringer Ingelheim for a collaboration on functional investigations of FKBP5. Otherwise, the authors declare that they have no competing interests.

Ethics approval and consent to participate/publish The ITU research protocol has been approved by the Coordinating Ethics Committee of the Helsinki and Uusimaa Hospital District (approval date: 06.01.2015, reference number: 269/13/03/00/09). The PREDO study protocol was approved by the Ethics Committee of Obstetrics and Gynaecology and Women, Children and Psychiatry of the Helsinki and Uusimaa Hospital District and by the participating hospitals. The BET study was approved by the Ethics Committee of the Charité-Universitätsmedizin, Berlin, Germany (EA2-149-07). In all studies, participants signed a written informed consent form.

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