CHARLES UNIVERSITY

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PHYSIOLOGICAL AND PHARMACOLOGICAL ASPECTS OF TRYPTOPHAN AND SEROTONIN HOMEOSTASIS IN THE FETOPLACENTAL UNIT

Doctoral Dissertation

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STATEMENT OF AUTHORSHIP

I hereby declare that I am the sole author of this thesis. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the thesis itself. All the literature and other resources from which I drew information are listed in the bibliography. The work has not been used to get another or the same title.

In Hradec Králové Mgr. Rona Karahoda

Date: …… ……...

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ABSTRACT

The placenta is an ephemeral organ inevitable for the successful course of pregnancy. As the main link between the mother and the fetus, the placenta fulfills numerous roles during gestation, including endocrine, transport, and immunoprotective processes. Proper functioning of the placenta is critical for the normal growth and development of the embryo/fetus. Importantly, the latest research has associated perturbations of maternal conditions (such as pharmacotherapy, malnutrition, diseases, stress, or inflammation) with alterations of the trophoblasts' endocrine, transport, and metabolic functions. Of note is the placental utilization of the essential amino acid tryptophan, suggested as a potential mechanism contributing to fetal programming of adulthood diseases. Tryptophan flux along the serotonin and kynurenine pathways generates metabolites with neuroactive, immunosuppressive, and antioxidant properties. Current literature suggests that fine-tuning of tryptophan metabolite concentrations in the fetoplacental unit is crucial for successful pregnancy outcome. Nonetheless, a comprehensive characterization of the enzymes and transporters involved in the metabolism/transport of tryptophan, serotonin, and kynurenines is still lacking. Moreover, controversies remain in the regulation of serotonin homeostasis in the fetoplacental interface.

On these grounds, the aims of this thesis were manifold and included: 1) detailed assessment of placental serotonin and kynurenine pathways during gestation in humans and rats, 2) evaluation of contribution of fetal organs (brain, intestine, liver and lungs) to the prenatal tryptophan metabolism, 3) characterization of serotonin handling in human and rat term placenta, and 4) effect of antidepressants on the placental serotonin system. A wide range of methodological approaches was utilized including *in vitro* transport assays, *in situ* perfusion of rat term placenta, isolation of membrane vesicles and primary trophoblast cells from human term placenta, gene expression analysis by Quantitative- and Droplet Digital PCR analysis, protein expression by western blotting, and metabolic activity of rate-limiting enzymes.

We report that the placental homeostasis of tryptophan is subject to strictly regulated developmental changes during pregnancy. We show that placental production of kynurenine increases during pregnancy, with a low contribution of other fetal organs. On the other hand, placental tryptophan metabolism to serotonin is crucial in early-to-mid-gestation, with a subsequent switch to fetal brain and intestine serotonin synthesis. We further provide the first evidence that human and rat term placenta extract fetal-derived serotonin via the organic cation transporter 3 (OCT3). Correspondingly, increased expression and function of serotonin-

degrading enzyme (MAO-A) and uptake transporters (SERT and OCT3) at term indicate efficient placental clearance of this monoamine, likely to prevent hyperserotonemia in the fetoplacental unit. We demonstrate that this orchestration between metabolizing enzymes and transporters is disrupted by antidepressants, which might at least partly explain the poor outcomes upon antidepressant use in pregnancy.

ABSTRAKT

Placenta je dočasný orgán, zajišťující spojení mezi matkou a plodem. Po dobu těhotenství vykonává řadu funkcí, včetně endokrinních, transportních a imunoprotektivních, které jsou zcela zásadní pro zdárný průběh gestace, normální růst a vývoj embrya/plodu. Nejnovější výzkumy poukazují na spojitost mezi endogenními (např. onemocnění, stres nebo zánět) a exogenními (např. farmakoterapie) faktory a změnami ve fyziologických funkcích placentárních trofoblastů. Příkladem může být narušení homeostázy látek s neuroaktivními, imunosupresivními nebo antioxidačními vlastnostmi. To může vyústit v nesprávné programování plodu a s tím spojené vyšší riziko závažných onemocnění v dospělosti. Jedním ze zdrojů takových metabolitů je esenciální aminokyselina, tryptofan. Je známo, že metabolismus tryptofanu probíhá serotoninovou a kynureninovou cestou, nicméně komplexní charakterizace enzymů a transportérů ovlivňujících placentární homeostázu tryptofanu, serotoninu a kynureninu je stále nedostatečná.

V rámci řešení této disertační práce jsme se tedy soustředili na studium: 1) změn serotoninové a kynureninové dráhy během těhotenství v placentě, 2) podílu fetálního mozku, střeva, jater a plic v prenatálním metabolismu tryptofanu, 3) schopnosti placenty vychytávat serotonin z fetální cirkulace a 4) účinku antidepresiv na placentární serotoninový systém. Byla použita široká škála metodických přístupů, zahrnujících *in vitro* transportní experimenty, *in situ* duální perfúze potkaní placenty, *ex vivo* akumulační experimenty, izolace membránových vezikul a primárních buněk trofoblastu z lidské placenty, analýzy absolutní a relativní genové exprese pomocí ddPCR a qRT-PCR, analýzy exprese proteinů pomocí western blotu a funkční analýzy klíčových enzymů.

Naše výsledky prokazují, že placentární homeostáza tryptofanu podléhá během těhotenství přísné regulaci. Placentární produkce kynureninu se v průběhu gravidity zvyšuje, nicméně další fetální orgány ke zvýšení produkce kynureninu velkou měrou nepřispívají. Na druhou stranu, placentární syntéza serotoninu je důležitá převážně v první polovině těhotenství; ve druhé polovině dochází k poklesu placentární produkce serotoninu, která je postupně nahrazována syntézou v mozku a střevě plodu. Z hlediska udržování hladin serotoninu ve fetoplacentární jednotce se ukázal být zásadní transportér pro organické kationty 3 (OCT3) lokalizovaný na bazální straně trofoblastu. Serotoninový transportér (SERT) naopak vychytává serotonin z maternální strany. Zvýšená exprese a funkce obou těchto placentárních transportérů a enzymu (MAO-A) ke konci těhotenství naznačuje účinnou extrakci a metabolickou degradaci

serotoninu placentou. Jedná se pravděpodobně o ochranný mechanismus proti hyperserotonemii ve fetoplacentární jednotce. V navazující studii jsme dále prokázali, že placentární clearance serotoninu je výrazně narušena antidepresivy; tento poznatek může alespoň částečně vysvětlovat nežádoucí účinky antidepresiv na vývoj a programování plodu.

LIST OF ABBREVIATIONS

5-HIAA - 5-hydroxyindoleacetic acid

5-OH-TRP - 5-hydroxytryptophan

AAT - System A amino acid transporter

ABC - ATP-binding cassette

ADHD - Attention deficit hyperactivity disorder

ATP - Adenosine triphosphate

BCRP - Breast Cancer Resistance Protein

BH4 - Tetrahydrobiopterin

BM - Basal membrane

CNS - Central Nervous System

CTBs - Cytotrophoblasts

DOHaD - Developmental Origins of Health and Disease

eCTBs - Endovascular trophoblasts

GLUT - Glucose transporter

hCG - Human Chorionic Gonadotrophin

iCTBs - Interstitial cytotrophoblasts

IDO - Indoleamine 2,3-dioxygenase

KYNA - Kynurenic acid

LAT - System L amino acid transporter

MAO - Monoamine oxidase

MDCKII - Madin-Darby canine kidney

MHC - Major Histocompatibility Complex

MRP - Multidrug Resistance-associated Proteins

mTOR - Mechanistic target of rapamycin

MVM - Microvillous membrane

NAD^+ - Nicotinamide adenine dinucleotide

NET - Norepinephrine transporter

NMDA - N-methyl-D-aspartate

OCT3 - Organic cation transporter 3

P-gp - P-glycoprotein

PTS - 6-pyruvoyltetrahydropterin synthase

QUIN - Quinolinic acid

SERT - Serotonin transporter

SLC - Solute carrier

SNRIs - Serotonin and norepinephrine reuptake inhibitors

SPR - Sepiapterin reductase

SRIs - Serotonin reuptake inhibitors

SSRIs - Selective serotonin reuptake inhibitors

STB - Syncytiotrophoblast

TDO - Tryptophan 2,3-dioxygenase

TPH - Tryptophan hydroxylase

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1 INTRODUCTION

The placenta is a unique organ serving as the main link between the mother and the fetus. Placental development during nine months of pregnancy is rapid, with the placenta continuously changing its structure and functions [1]. Considering its complex position as the maternal-fetal interface, the placenta undertakes various functions to ensure successful fetal development and pregnancy outcome. Notably, certain insults during pregnancy, including pharmacotherapy, inflammation, malnutrition, or environmental toxins, can alter the placenta's normal functioning [2]. Numerous epidemiological studies have demonstrated that placental adaptations to these insults allow the fetus to survive, but at the cost of permanently impairing its physiology and development [3-5]. Subsequently, the fetus is predisposed to an increased risk of mental, metabolic, or cardiovascular disorders later in life, a phenomenon known as fetal programming or developmental origins of health and disease (DOHaD) [6, 7]. While the molecular mechanisms involved in fetal programming are largely unknown, several possible pathways have been suggested.

Of interest is the placental tryptophan metabolism, which *inter alia* gives rise to serotonin, melatonin, and kynurenines. These metabolites are associated with several functions, including immunosuppression, neuroactivity, antioxidative properties, and NAD^+ synthesis [8]. Current literature suggests that optimal levels of tryptophan metabolites in the fetoplacental unit are crucial for proper placenta function, fetal development, and programming [9]. Considering the various roles of tryptophan metabolites during the prenatal period, it is essential to delineate the mechanisms involved in placental tryptophan metabolism and/or transport. Additionally, knowledge on the regulation and interplay of serotonin and kynurenine pathways during gestation could provide a better understanding on the significance of a specific pathway at a certain point in pregnancy. Importantly, studying potential perturbations (such as pharmacotherapy in pregnancy) affecting the function of placental metabolizing enzymes and/or transporters involved in tryptophan homeostasis is critical in identifying molecular mechanisms affecting fetal programming. As most tryptophan metabolites are neuroactive, these mechanisms may alter neurodevelopmental processes in the developing embryo and contribute to the developmental origins of neurobehavioral and psychiatric disorders [9].

2 THEORETICAL BACKGROUND

2.1 Placental types and structure

An extraordinary structural diversity exists in the development of the placenta throughout mammalian species. Several classifications are used to categorize the placenta. They include the origin of fetal membranes, placental shape, histological structure of the maternal-fetal interface, type of maternal-fetal interdigitation, trophoblast invasiveness, and decidual cell reaction [10]. The type of maternal-fetal interdigitation describes the geometrical pattern by which the maternal and fetal tissues are arranged to form the placenta. The most sophisticated type is represented by the labyrinthine arrangement in rodents and lower primates, in which maternal blood circulates through web-like channels within the fetal syncytiotrophoblast [11]. On the other hand, in humans, the chorion forms tree-like villi in direct contact with maternal tissues, which is known as the villous type of placentation [12].

Another important classification system is the Grosser classification describing the layers comprising the interhaemal area [13]. Rodent and human placenta are of the hemochorial type where the chorionic surface is in direct contact with maternal blood. According to the number of trophoblast layers, this placental type has further been divided into hemotrichoral (three layers of trophoblast, as found in rodents), hemodichoral (two trophoblastic layers, found in beaver and early human) and hemomonochorial (typical of human placenta) [10, 11, 14].

2.1.1 Development of the human placenta

In the first weeks of pregnancy, multiple cell division stages give rise to trophectoderm and the inner cell mass. Trophectoderm, the precursor of placental cells, interacts with the uterine epithelium allowing implantation. On the other hand, the inner cell mass gives rise to the embryo. Implantation of trophectoderm allows the generation of mononucleated cytotrophoblast cells (CTBs), which then differentiate into highly specialized cells undertaking various functions. Specifically, differentiation by fusion gives rise to multinucleated syncytiotrophoblast (STB) in the anchoring villus. The STB serves as a mechanical barrier between maternal and fetal circulation via the maternal-facing microvillous (MVM) and fetal-facing basal membranes (BM), respectively. Subsequent vascularization of the floating villi establishes a maternal-fetal exchange interface and contributes to placenta development. On the other hand, CTB proliferation and migration to decidua generate extravillous trophoblast cells. A subset of these cells, interstitial trophoblasts (iCTBs), invade decidua and establish the interaction with uterine cells, whereas endovascular trophoblasts (eCTBs) replace endothelial

cells in the maternal spiral arteries, aiding proper oxygen and nutrient delivery to the fetus (Figure 1) [1, 15, 16].

The mature placenta is surfaced by the chorionic plate, facing the fetus and the basal plate, adjacent to the maternal endometrium. In between is a cavity of intervillous space where around 30-40 villous trees, branching from the chorionic plate, are dispersed. The chorionic villi are bathed into maternal blood, released at the openings of maternal spiral arteries through the basal plate. The villous trees' final branches are highly vascularized by a fetal capillary network, with the endothelium being in close contact with the trophoblast layer. Thus, this represents the primary site of maternal-fetal exchange, composed of multiple independent units (Figure 1) [17].

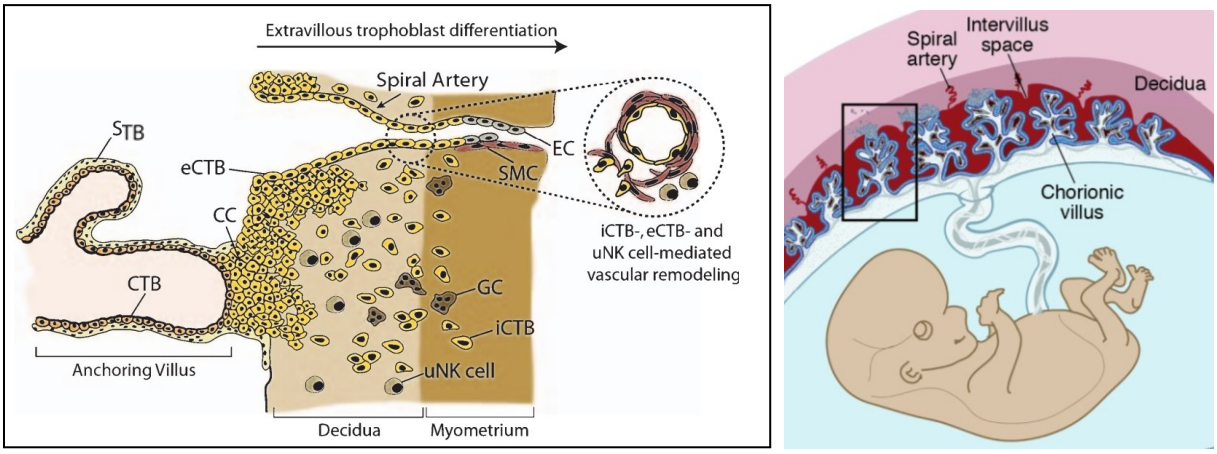

Figure 1. Trophoblast differentiation and human placenta development. Left panel: Mononucleated cytotrophoblast cells in the anchoring villus give rise to the differentiated syncytiotrophoblast layer, which forms the placental barrier between the mother and fetus responsible for the transport of nutrients and hormone production. A population of CTBs migrates to decidua giving rise to invasive trophoblasts or endovascular trophoblasts, promoting uterine invasion and vascular remodeling, respectively. Right panel: Structure of fetoplacental interface, depicting chorionic villi perfusion by maternal blood leaving the decidual spiral arteries into the intervillous space. Adopted and modified from Pollheimer and Knöfler, 2012 [18] and Maltepe et al., 2010 [19].

Abbreviations: CC - cell column trophoblasts, CTBs - Cytotrophoblasts, EC - endothelial cells, eCTBs - Endovascular trophoblasts, GC - giant cells, iCTBs - Interstitial cytotrophoblasts, SMC - smooth muscle cells, uNK - uterine natural killer cells.

2.2 Experimental models to study placental biology

Ethical and technical constraints often limit placental investigation directly in humans under *in vivo* conditions. Therefore, it is essential to collect experimental data via alternative methods, and often a combination of several experimental models (Figure 2) is used to confirm the findings. These techniques have specific pros and cons [20], so the acquired data must be treated cautiously due to the complexities and potential confounding factors involved.

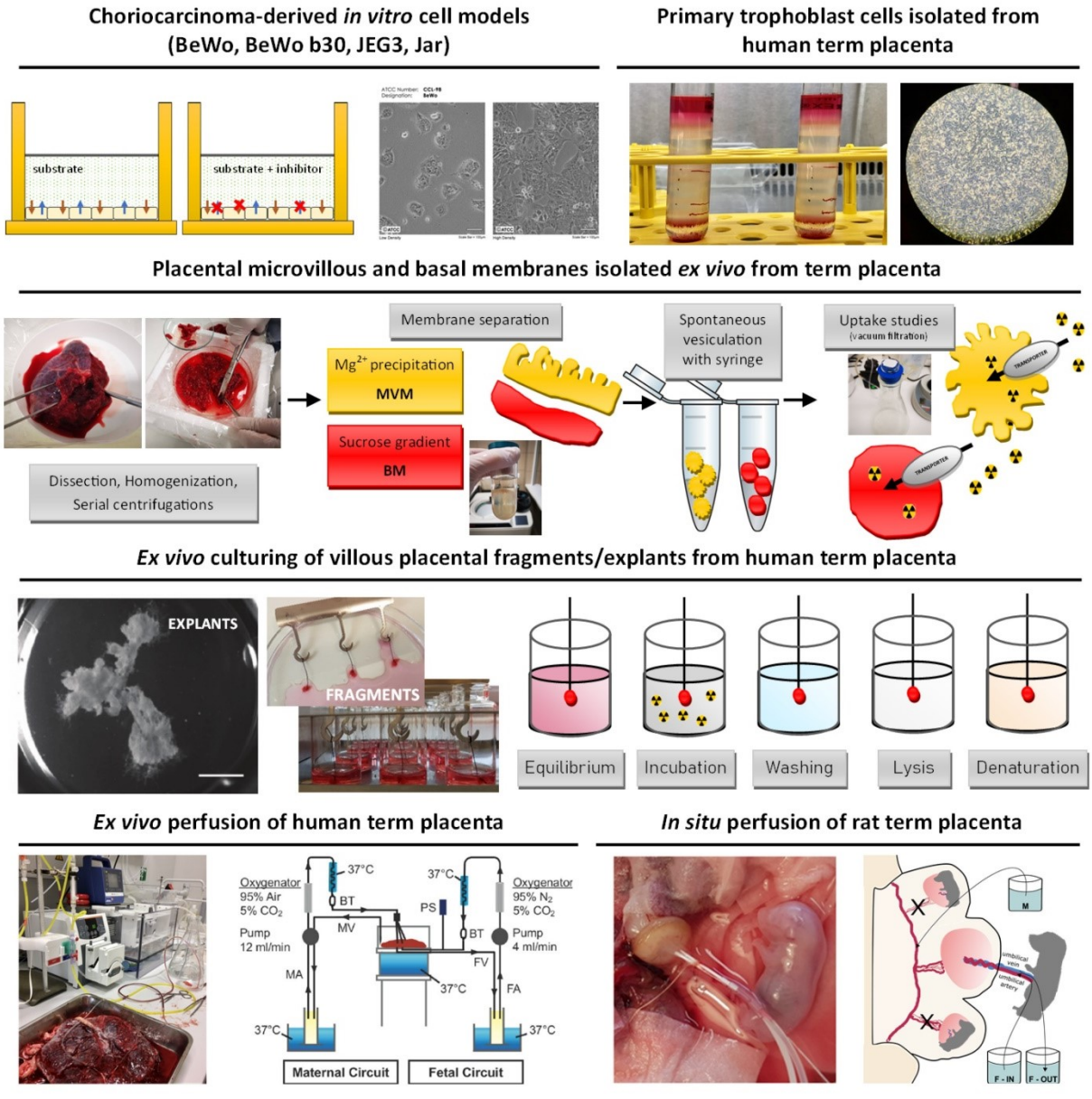

Figure 2. Summary of experimental models (human and rodent) used to study placental physiology, pathology, and pharmacology. The representative picture of villous explants culture was obtained from Mannelli et al. [21], whereas the schematic depiction of human placenta perfusion was adopted from Grafmüller et al. [22].

2.2.1 Human placenta models

Several human placental-derived models have been developed throughout the past decades to investigate placental physiology, pathology and pharmacology. Nonetheless, as placental tissue availability is most feasible upon delivery, it largely restricts the research to the very end of pregnancy. Even so, the *ex vivo* perfused human term placenta is extensively used in the investigation of nutrient, drug, and nanoparticle transport, potential interactions in the placental transporter systems, and analysis of biotransformation enzymes [23-25]. Similarly, isolation of membrane vesicles (MVM and BM) from human term placenta, via differential centrifugation steps, Mg^{2+} precipitation, and sucrose gradient, is particularly useful in high-throughput screening of transporter-mediated mechanisms on separate placental membranes [26]. However, the isolated membranes are devoid of regulatory factors which, under physiological conditions, would contribute to transporter function; thus, extrapolation to *in vivo* situation is rather difficult [20].

Additionally, the human placenta is used to isolate primary trophoblast cells via trypsin digestion and Percoll gradient centrifugation, which in culture spontaneously fuse to form STB [27]. This model represents physiological trophoblast and can be used to study different aspects of placental metabolism, transport, or pathology. Recent work has highlighted the advantage of isolated primary trophoblast cells when compared to placental cell lines derived from choriocarcinoma, such as BeWo, JEG-3, and Jar [28]. While easy to work with, these placental cell lines do not reflect physiological behavior of trophoblast cells and have been shown to express a different enzyme/transporter portfolio compared to primary trophoblast cells. In addition a more pronounced effect of differentiation upon the use of differentiation-inducing agents was reported [28]. Lastly, villous fragments [29] and explants [30] can be isolated from the human placenta, with the explant model further maintained in culture for up to 7 days [21]. These models are favorable since tissue integrity is maintained and used for different purposes, including transport, metabolism, and toxicity assays.

2.2.2 Animal models

Animal models have been essential in advancing our understanding of the prenatal environment. Long-term administration of several agents (e.g. drugs, inflammatory agents, toxins) in pregnant animals has allowed in-depth evaluation of placental functions and estimation of fetal exposure and toxicity [31, 32]. Moreover, *in situ* perfused animal placenta (mouse, rat, sheep) shares similar advantages to human placenta perfusion [33, 34], with sample

availability being more attainable. Lastly, the use of innovative imaging systems to study fetal/placental development has been critical in fetal programming studies [35]. Nonetheless, when using animal models, extreme caution should be taken to consider interspecies differences. In particular, concerning tryptophan metabolism, significant differences exist between different mouse strains [8]. In this aspect, the Wistar rat has been recommended as the most suitable model for placental tryptophan metabolism in health and disease [8, 36].

2.3 Placental functions

The placenta is the first and largest fetal organ which plays more diverse functions than any other organ. Specifically, it serves as a digestive, excretory, respiratory, endocrine, and immune system [37]. Naturally, pregnancy is characterized as an immunological challenge since the fetus is genetically distinct from the mother. Many mechanisms have been suggested to play a role in modulating the maternal immune system [38], among others the restriction and modulation of leukocytes [39], the lack of classical MHC class II molecules in the trophoblast [40], and placental tryptophan utilization [41, 42].

The key structure implicated with placental functions is the STB layer due to its critical position in the maternal-fetal interface and high metabolic rate [43]. For a long time, it was believed that as pregnancy proceeds, the CTB layer disappears [14], however, the latest research has shown an increasing number of CTBs at term [44]. Moreover, Kolahi et al. recently demonstrated that undifferentiated CTBs are the most metabolically active cells in the human term placenta, with a high fuel flexibility level [45]. These findings suggest that CTBs may also substantially contribute to global placental metabolism during gestation and call for future studies to focus on CTB's role in placental functions.

2.3.1 Endocrine function: Main placental hormones and their function

As a highly active endocrine organ, the placenta secretes various hormones into the maternal and fetal circulation, thus modulating their physiology and mediating maternal adaptations during pregnancy. Metabolic cues act upon maternal cardiovascular, respiratory, hematological, nervous, immune, and metabolic systems causing alterations in size, morphology, function, and responsiveness of these tissue systems [46]. Essential placental hormones include human chorionic gonadotrophin (hCG), prolactin and growth hormone family, steroid hormones, and neuroactive hormones [37, 46, 47].

hCG is one of the most important pleiotropic hormones during pregnancy. It stimulates progesterone production, promotes syncytialization, angiogenesis, and immunotolerance, supports trophoblast invasion, and is implicated with endometrial receptivity and embryo implantation [37]. On the other hand, the prolactin and growth hormone family consists of prolactin, placental lactogens, prolactin-like hormones, proliferins, and growth hormone [46], chiefly implicated in mediating maternal metabolic adaptations via regulation of maternal insulin production and sensitivity. Additionally, they affect maternal appetite and body weight, mammary gland function, and maternal behavior [37, 46]. Leptin, a peptide hormone also synthesized by the placenta, affects placental functions, including trophoblast invasion, embryo implantation, and immunomodulation [37].

Likewise, steroidogenesis in the maternal-placental-fetal unit plays a pivotal role in pregnancy maintenance and fetal growth and development. Apart from ensuring steroid transfer and communication between maternal and fetal compartments, placenta also maintains steroid homeostasis by its own synthesis and metabolism of cholesterol, sex hormones, and corticosteroids. Specifically, the placenta secretes a high amount of progesterone and estrogens; on the other hand, it has been deemed incapable of androgen synthesis, thus rendering it dependent on fetal sources [47, 48]. Progesterone participates in immunotolerance [49], decidualization of the endometrium [50], regulates trophoblast invasion [51], and regulates insulin and glucose homeostasis [46]. Androgens are essential in modulating maternal vasculature, endothelial cell proliferation, and the development of sexual characteristics [52]. Additionally, androgens serve as precursors of estrogens, which are vital in promoting embryo implantation and angiogenesis [53], and maternal metabolic adaptation [46]. Concurrently, glucocorticoids regulate metabolic homeostasis, inflammatory and immune reactions, and the promotion of trophoblast proliferation and invasion [54].

Placenta also exerts neuroendocrine effects via the activity of several neuroactive hormones. Serotonin and melatonin, tryptophan-derived hormones, are synthesized within the placenta [55, 56] and impact the maternal and fetal brain and related neuroendocrine organs. Both hormones maintain maternal glucose homeostasis, support fetal organ development and programming [57, 58], regulate steroid synthesis [59-61], and are important for lactation [46]; melatonin further regulates circadian rhythmicity [62]. Other neuroactive hormones produced by the placenta include kisspeptins, affecting the maternal cardiovascular system [46], promoting trophoblast adhesion, and inhibiting trophoblast invasion and angiogenesis [37].

Abnormal production of placental hormones affects physiological processes during gestation. This may interfere with proper placental and fetal functions/development, leading to several pathologies, including but not limited to preeclampsia, intrauterine growth restriction, and gestational diabetes mellitus [37]. In addition, hormonal disbalance in the fetoplacental unit may result in improper "wiring" of fetal organs and thus contribute to DOHaD.

2.3.2 Transport function: Role of transporters in the placental transfer of nutrients and pharmaceuticals

The developing fetus is dependent on the maternal supply of nutrients while at the same time, fetal waste products are transported back to the maternal circulation. Exchange of nutrients and waste products between the mother and fetus across the placenta occurs mainly via passive diffusion and/or transporter-mediated mechanisms. Diffusion is particularly important for the exchange of oxygen, and it is assumed that the requirements for oxygen exchange are the principal drivers of placental architecture [17]. On the other hand, diffusion of small lipophilic molecules is mainly dependent on the concentration gradient, which is influenced by the blood flow rate across the membrane [17].

The placental STB layer is equipped with a battery of transporters localized in the maternal-facing MVM and/or fetal-facing BM. These transporters facilitate the transfer of nutrients across the placenta and control the transplacental disposition of many drugs (Figure 3) [63]. Two transporter classes are recognized: the ATP-binding cassette (ABC) superfamily and the solute carrier (SLC) transporter family. Of ABC transporters, three members are mainly characterized as substantial in the placenta, namely P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein 2 (MRP2). They actively pump their substrates out of the trophoblast cells into the maternal circulation, using ATP as energy source [64, 65]. As such, they play a critical role in fetal protection against drugs and other toxins.

On the other hand, SLC transporters are predominantly facilitative or secondary-active, transporting hydrophilic/charged molecules into the trophoblast cells [66, 67]. Several members have been described and include amino acid transporters [best characterized: System L (LAT) and A (AAT) transporters] [68], glucose transporters (GLUTs) [69], monoamine transporters [serotonin (SERT) and norepinephrine (NET) transporters] [70, 71], organic cation transporters (OCTs; specifically OCT3 [72]), members of organic anion transporters [63], carnitine transporters [66], nucleoside transporters [73], organic anion transporting polypeptides [63] and

multidrug and toxin extrusion proteins [74]. Members of the SLC family can be specific or polyspecific to their substrates, and apart from nutrients, they may transport a wide range of drugs and toxins. Thus, they represent potential targets of drug-drug and drug-nutrient interactions [75].

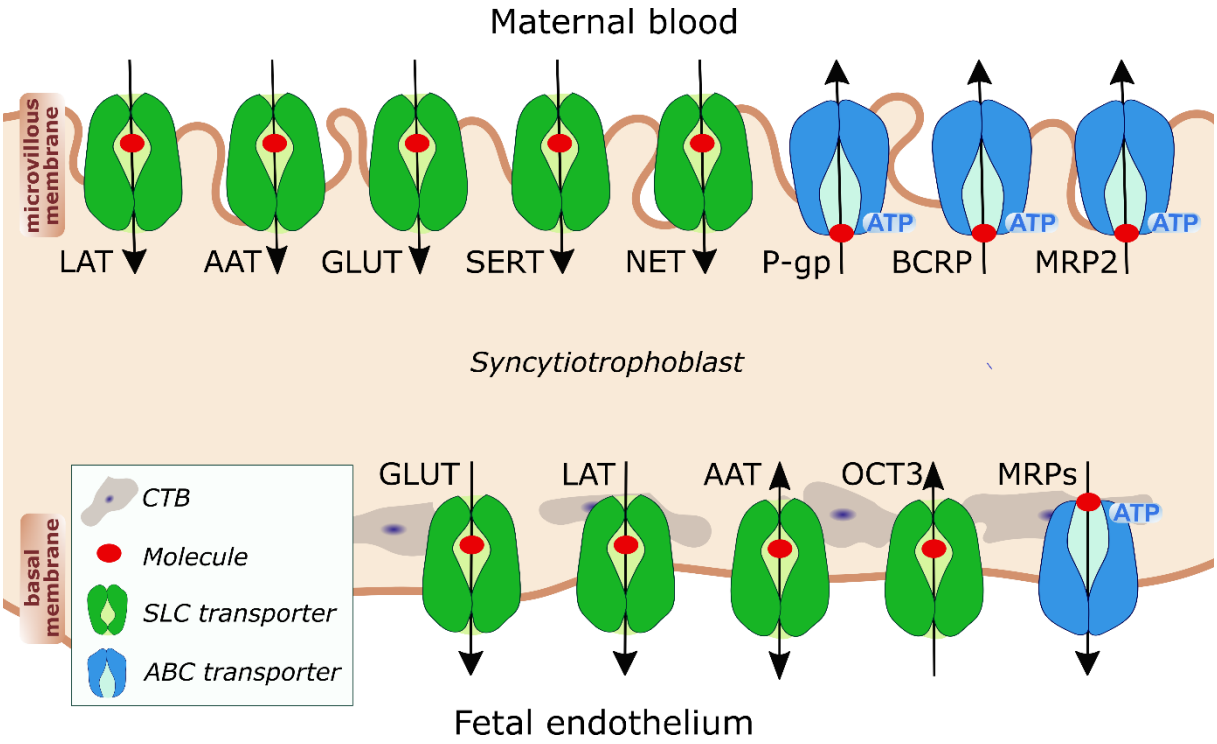

Figure 3. Schematic summary of main nutrient and drug transporters in the placenta, localized in the maternal-facing microvillous membrane and fetal-facing basal membrane. ABC transporters function as protective efflux transporters using ATP as an energy source, whereas SLC transporters mainly mediate the influx of various molecules via facilitated diffusion.

Abbreviations: AAT - System A amino acid transporters, ABC - ATP-binding cassette, ATP - adenosine triphosphate, BCRP - breast cancer resistance protein, CTB - cytotrophoblast, GLUT - glucose transporter, LAT - System L amino acid transporter, MRP2 - multidrug resistance-associated protein 2, NET - norepinephrine transporters, OCT3 - organic cation transporter 3, P-gp - P-glycoprotein, SERT - serotonin transporter, SLC - solute carrier.

2.4 Role of the placenta in fetal programming of adulthood diseases; underlying mechanisms

The last three decades have been remarkable in shedding light on the importance of the prenatal environment, not only for the fetus's proper development but also for the programming of adulthood diseases. The DOHaD concept dates to 1993 when Barker et al. reported a link between maternal undernutrition at different stages of pregnancy with abnormal fetal growth

and permanent changes in fetal physiology, structure, and metabolism. Ultimately, the authors postulated that adaptations to these conditions might lead to metabolic abnormalities, cardiovascular, and CNS diseases in adult life [6]. Since then, several epidemiological studies [3-5] have shown that the intrauterine environment is closely linked to the risk of a wide range of adult diseases, and research has highlighted a significant role of placental function in the overall predisposition [76, 77].

While detailed molecular mechanisms of fetal programming are yet to be fully elucidated, it is well accepted that fetal programming occurs through various regulatory, metabolic, and endocrine pathways mediating the flow of information between the mother and fetoplacental unit [76]. One example is the altered maternal nutrition state, which exerts specific mechanisms within the placenta, altering nutrient and oxygen supply, hormonal secretion, and nutrient-sensing signaling pathways [2]. In this respect, the mechanistic target of rapamycin (mTOR) has been suggested as a molecular mechanism for placental nutrient sensing [2] (Figure 4). Specifically, by integrating signals of nutrient load (including glucose, amino acids, fatty acids, and oxygen levels) and/or hormonal status in the maternal circulation, it responds by up- or down-regulating placental nutrient transporters [78-80]. Altered fetal nutrient availability has been associated with pregnancy conditions such as intrauterine growth restriction [81] and large for gestational age babies [80]. These conditions are in turn associated with increased risks of metabolic and cardiovascular disorders in adulthood [2]. Thus, maternal nutritional status during pregnancy, and placental nutrient delivery to the developing fetus, are critical in the developmental programming of physiological processes.

Another important mechanism of fetal programming is glucocorticoid homeostasis in the placenta (Figure 4). As the fetus is incapable of cortisol synthesis, it depends on maternal supply [82]. Nonetheless, as the hypothalamic-pituitary-adrenal axis programming is particularly sensitive to glucocorticoids, cortisol levels in the fetus must be tightly controlled. This is ensured by the activity of placental 11-beta hydroxysteroid dehydrogenase 2, which deactivates cortisol to cortisone [47, 82]. However, this enzyme's expression and activity are prone to alteration by factors such as pharmacotherapy, polymorphisms, stress, dietary restriction, hypoxia, or inflammation. The involvement of this pathway in fetal programming was demonstrated as early as 1993 when Edwards et al. showed a link between impaired glucocorticoid barrier in the placenta and adult hypertension [83].

More recent work has highlighted the role of prenatal environment in the programming of CNS disorders including depression, ADHD, psychiatric or autism spectrum disorders. Specifically, maternal stress, infection, or malnutrition have been significantly linked to the risk of developing schizophrenia and autism in adults [9, 84-86]. Several perspectives have emerged to account for the mechanisms by which prenatal events induce changes leading to mental health disorders. In this regard, serotonin and kynurenine pathways of tryptophan metabolism have recently been described in the STB and suggested as a novel alley for the developmental origins of mental diseases (Figure 4) [9]. This is due to the neuroactive nature of several metabolites generated along these two pathways (see Chapter 2.5). Notably, the expression and activity of the rate-limiting enzymes of tryptophan metabolism in the placenta may be affected by maternal inflammation, stress, depression, polymorphisms, and xenobiotics [87, 88]. These factors may alter tryptophan catabolism and disbalance the levels of tryptophan metabolites in the fetoplacental unit, eventually affecting fetal brain development and programming.

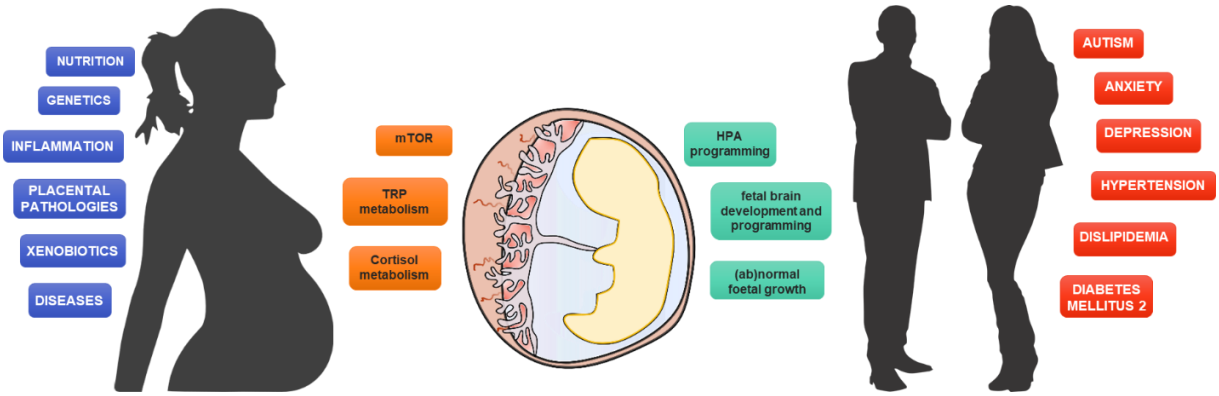

Figure 4. Proposed mechanisms involved in the maternal-placental-fetal interface and fetal programming. Disturbed maternal conditions in the prenatal period lead to altered placental functions, which affect fetal development and predispose the newborn/offspring to adult-onset disorders.

Abbreviations: HPA - hypothalamic–pituitary–adrenal axis, mTOR - mechanistic target of rapamycin, TRP - tryptophan

2.5 Placental tryptophan metabolism

Tryptophan is an essential amino acid supplied via dietary intake of foods including meat, fish, milk, eggs, vegetables, nuts, soybeans, sesame, and sunflower seeds. Apart from protein synthesis, tryptophan is metabolized to several active metabolites and two pathways are

recognized in the placenta: a) the kynurenine pathway and b) the serotonin pathway (Figure 5) [87].

Extensive literature research identified several methods used to study placental tryptophan biology. They include a variety of human and animal models such as clinical cohort studies [89, 90], analyses of tissue homogenates of human [91] or animal placentas [88, 92, 93], perfused mouse placenta [55, 88, 92], and placental villous explants [30, 90] (Figure 2).

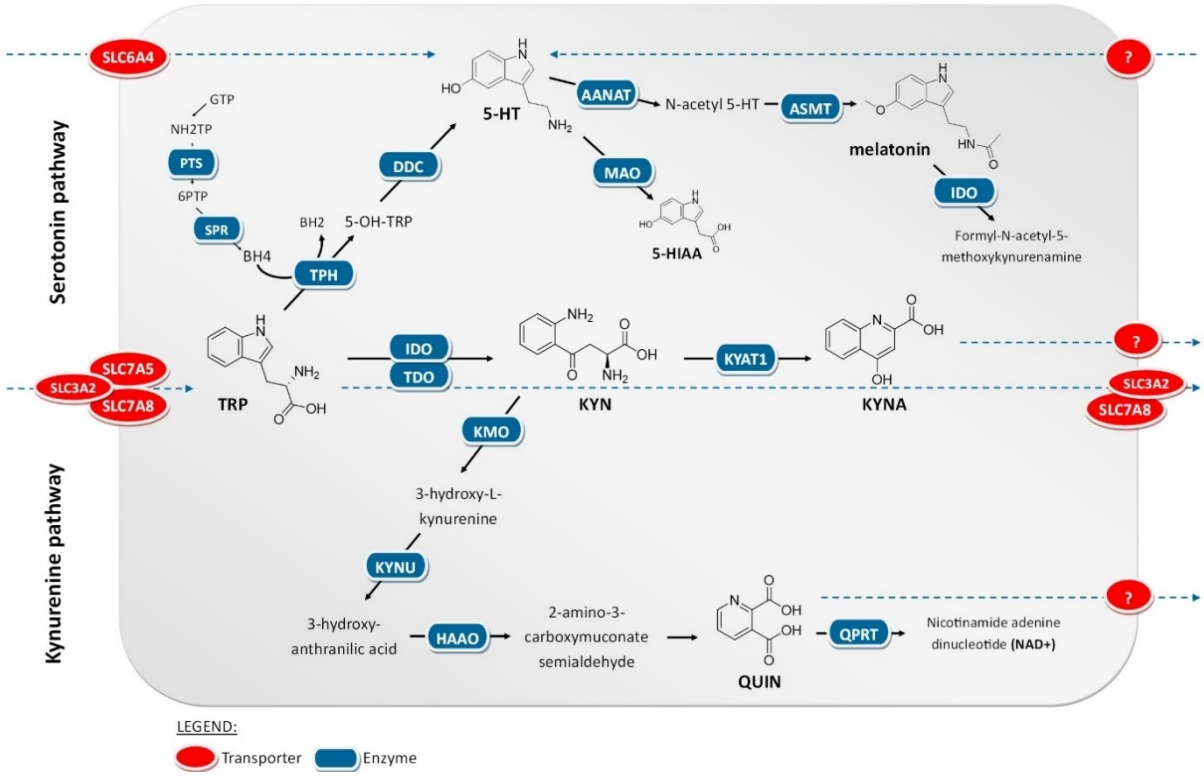

Figure 5. Schematic representation of placental metabolism of tryptophan. The serotonin pathway gives rise to neuroactive metabolites, including serotonin and melatonin involved in placentation, fetal growth and development, and circadian rhythmicity. Kynurenine pathway generates metabolites such as kynurenine, kynurenic acid (KYNA), and quinolinic acid that apart from being neuroactive in nature, they are implicated in immunosuppression and redox reactions.

Abbreviations: 5-HT - serotonin, 5-OH-TRP - 5-hydroxytryptophan, 6PTP - 6-pyruvoyl-tetrahydrobiopterin, AANAT - aralkylamine N-acetyltransferase, ASMT - acetylserotonin O-methyltransferase, BH4 - tetrahydrobiopterin, GTP - guanosine triphosphate, HAAO - 3-hydroxyanthranilate 3,4-dioxygenase, IDO - indoleamine 2,3-dioxygenase, KMO - kynurenine 3-monooxygenase, KYAT1 - kynurenine aminotransferase 1, KYN - kynurenine, KYNA - kynurenic acid, KYNU - kynureninase, MAO - monoamine oxidase, NH2TP - 7,8-dihydroneopterin triphosphate, PTS - 6 pyruvoyltetrahydropterin synthase, QPRT - quinolinate phosphoribosyltransferase, QUIN - quinolinic acid, SPR - sepiapterin reductase, TDO - tryptophan 2,3-dioxygenase, TPH - tryptophan hydroxylase, TRP - tryptophan

2.5.1 Kynurenine pathway

Tryptophan metabolism in mammals occurs predominantly along the kynurenine pathway using indoleamine 2,3-dioxygenase-1/2 (IDO1/2) and tryptophan 2,3-dioxygenase (TDO) as the rate-limiting enzymes [87]. In the placenta, the expression of IDO1 has been extensively investigated, showing minimal expression in the first trimester and upregulation towards term [90, 94-97]. Nonetheless, its localization in the placenta remains contradictory; while some older studies report IDO1 localization in villous or extravillous trophoblasts [94, 98, 99], Blaschitz et al. have most recently shown exclusive expression of IDO1 in endothelial cells where it contributes to immunosuppression and placental tone relaxation [96].

Kynurenine is further metabolized to kynurenic acid (KYNA) and quinolinic acid (QUIN), metabolites with neuroactive properties acting on the N-methyl-D-aspartate (NMDA) receptor in the CNS [100, 101]. While the importance of placental KYNA and QUIN is to date unknown, Manuelpillai et al. determined the placental expression of all enzymes involved in the kynurenine pathway [102]. On the other hand, recent studies in mouse term placenta report a minimal placental contribution to fetal KYNA levels [92, 103]. Additionally, kynurenine metabolites such as 3-hydroxykynurenine, anthranilic acid, and 3-hydroxyanthranilic acid have been reported to exert antioxidative and immunosuppressive action. In general, placental tryptophan metabolism along the kynurenine pathway is believed to play an essential role in allogeneic fetal rejection and is important for achieving immunotolerance for the fetus [8, 87].

2.5.2 Serotonin pathway

Tryptophan metabolism along the serotonin pathway is mediated by the rate-limiting enzyme tryptophan hydroxylase (TPH). TPH utilizes tetrahydrobiopterin (BH4) as a cofactor [104], giving rise to serotonin, an essential trophic factor early in gestation (Figure 5) [55]. In addition, serotonin is important for blastocyst implantation, placentation, and decidualization [105, 106]. Nonetheless, while the placenta has been deemed an organ controlling prenatal serotonin levels, serotonin's placental handling has been controversial in the current literature. Older studies presented the placenta as a barrier against maternal monoamines [107], whereas newer reports demonstrated maternal-to-fetal transport of serotonin via serotonin transporter (SERT) expressed in the MVM [70, 108, 109]. Interestingly, in a breakthrough study in 2011, Bonnin et al. further showed that at a precise time-window of pregnancy, the placenta synthesizes serotonin from maternal tryptophan and delivers it to the fetus for brain development [55]. This was later confirmed *in vitro* using primary trophoblast cells isolated from human term placenta

[91]. Placental supply of serotonin to the fetus is considered crucial since early in pregnancy the fetus is not capable of serotonin synthesis. Nonetheless, from mid-gestation onwards the fetus gains serotonin-synthesizing capacity utilizing maternally derived tryptophan [110, 111]. This suggests that at term placental supply of serotonin may no longer be necessary.

Notably, within the placenta, serotonin can further be metabolized to melatonin [56], involved in circadian rhythmicity, fetal growth, and placental function regulation [58, 112, 113]. The placenta also expresses substantial amounts of MAO-A, degrading serotonin to 5-hydroxyindole acetic acid (5-HIAA) [114-116]. Hyper- or hypo-serotonemia in the fetoplacental unit are detrimental for placental vasculature [117] and fetal development [118]. Thus, the expression and activity of key enzymes and transporters involved in serotonin handing in the fetoplacental unit must be tightly regulated during the whole period of gestation.

2.6 Pharmacotherapy in pregnancy; effect of antidepressant drugs on placental serotonin homeostasis

Pharmacotherapy in pregnancy is often necessary and inevitable for medical treatment of the mother, the fetus, or both [63]. Depression, a condition affecting up to 20% of pregnant women [119], has been associated with poor maternal and neonatal outcomes. Specifically, pregnant women with untreated depression are in a greater risk of alcohol/tobacco abuse or malnutrition [120]. Additionally, neonates born to depressed mothers are more likely to be delivered preterm, have a lower birth weight, exhibit social interaction impairment, and show differences in the developmental and emotional aspects [120]. Thus, the use of antidepressant drugs during pregnancy is recommended and has significantly increased in recent years.

Latest data estimate that approximately 13% of pregnant women are exposed to at least one antidepressant drug during pregnancy [121]. The most commonly prescribed antidepressants belong to the group of selective serotonin reuptake inhibitors (SSRIs): sertraline, citalopram, paroxetine, fluvoxamine, or fluoxetine [122] and serotonin and norepinephrine reuptake inhibitors (SNRIs): venlafaxine and duloxetine [123]. The mechanism of action of these drugs relies on the inhibition of SERT, increasing serotonin concentrations in the synapses of the CNS. However, lipophilic in nature, antidepressants cross biological membranes (including placenta) with ease, potentially distributing in the fetoplacental unit and affecting prenatal serotonin homeostasis [124].

Moreover, prenatal antidepressant use is linked to an increased risk of congenital and cardiac malformations [125], fetal pulmonary hypertension [126], gestational hypertension, and

preeclampsia [127]. Notably, associations between antidepressant use in pregnancy and a wide range of neurobehavioral sequelae (ADHD, autism, depression) has been shown [128-131]. While detailed molecular pathways have not been satisfactorily explained to date, alterations in serotonin handling in the fetoplacental unit have been suggested [132]. This can have consequences in the placental serotonin homeostasis, important for fetal development and placental functions (see Chapter 2.5.2). Mechanistically, it could contribute to a significant range of abnormalities during pregnancy, such as preterm delivery, pulmonary hypertension, intrauterine growth restriction, and neurobehavioral disturbances in infants [132, 133].

3 AIMS OF THE DISSERTATION THESIS

This study examined various aspects of tryptophan homeostasis in the fetoplacental unit in rats and humans. The aims of the thesis were manifold and included:

i. a detailed assessment of tryptophan flux along the serotonin and kynurenine pathways during gestation in human placenta,
ii. tryptophan catabolism in the fetoplacental unit during gestation in rat,
iii. characterization of serotonin homeostasis (i.e., transport, synthesis and degradation) in human and rat term placenta,
iv. effects of antidepressant drugs on the placental serotonin system.

4 RESULTS AND DISCUSSION

This dissertation thesis is organized as an annotated set of four research articles and one invited review (4.1). The main candidate is the first author of three articles, with two of them in the shared first-author position (4.3 and 4.4). Four of these articles are published in international journals with impact factor, and one article (4.5) has been submitted to an international journal with impact factor. The outlines of these publications and candidate's contribution is listed below.

4.1 Trophoblast: The central unit of fetal growth, protection and programming

Staud F, **Karahoda R**. *Int J Biochem Cell Biol.* 2018;105:35-40. **(IF = 3.25, Q1)**

In this invited review article, we discussed several aspects of placental biology. Specifically, we focused on the role of the trophoblast cells in placental and fetal development and the establishment of maternal-fetal communication. We considered placental cell origin, and differentiation of cytotrophoblast cells, highlighting the role played by the STB layer, iCTBs, and eCTBs. Further, we summarized the main autocrine/paracrine factors, signaling pathways, and transcription factors that regulate the differentiation of CTBs into villous and/or extravillous trophoblasts. One chapter describes placental functions, reviewing the endocrine, transport, and feto-protective roles the placenta plays throughout pregnancy. Finally, a special section is dedicated to fetal programming, where we reviewed the key placental mechanisms suggested to mediate prenatal programming of adult-onset diseases. Specifically, we discussed the role of mTOR signaling pathway, placental transport of glucose, amino acids and fatty acids, cortisol metabolism, and tryptophan metabolism along the serotonin and kynurenine pathways (Figure 6).

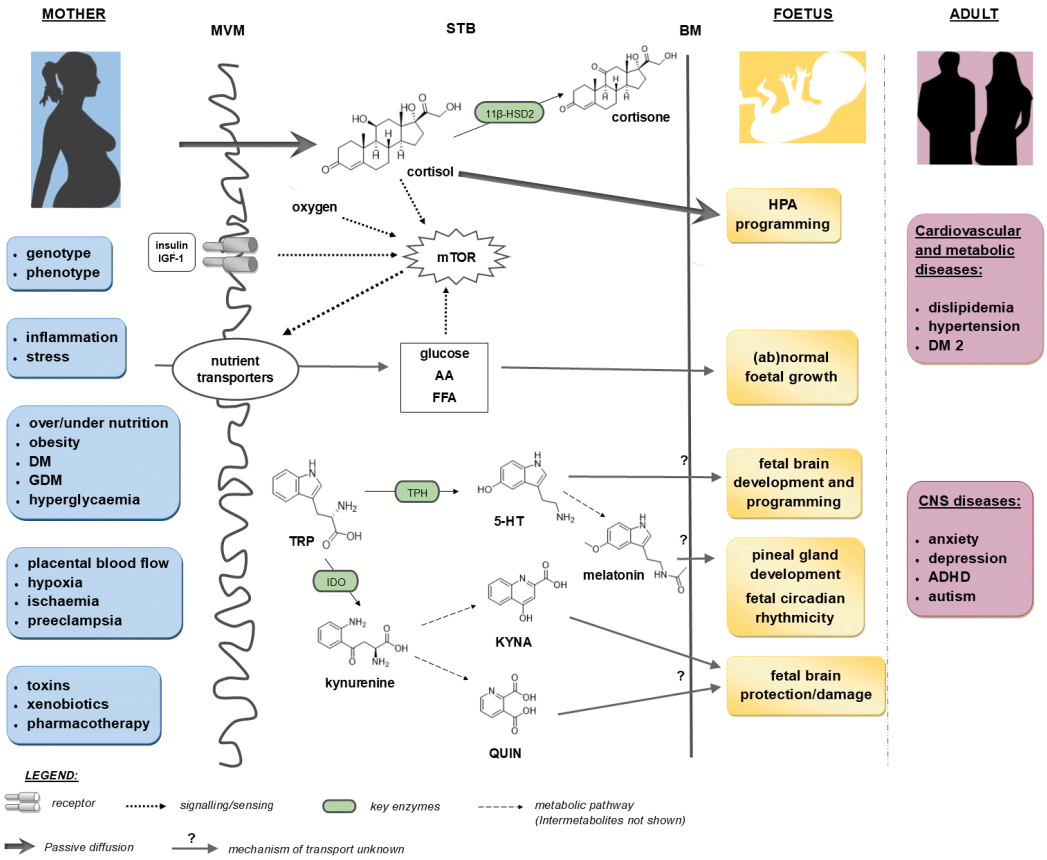

Figure 6. Graphical summary of the main placental mechanisms involved in fetal programming.

Abbreviations: 11β-HSD2 - 11beta-hydroxysteroid dehydrogenase, 5-HT - serotonin, AAs - amino acids, ADHD - attention-deficit/hyperactivity disorder, BM - basal, membrane, DM - diabetes mellitus, DM2 - DM type 2, FFAs- free fatty acids, GDM - gestational diabetes mellitus, HPA - hypothalamic-pituitary-adrenal axis, IDO - indoleamine 2,3-dioxygenase, IGF - insulin-like growth factor, KYNA - kynurenic acid, mTOR - mammalian target of rapamycin, MVM - microvillous membrane, QUIN - quinolinic acid, STB - syncytiotrophoblast, TDO - tryptophan 2,3-dioxygenase, TPH - tryptophan hydroxylase, TRP - tryptophan.

Candidate's contribution:

- Literature research and analysis, responsible for the "Cell origin and plasticity" part, preparation of figures, writing and revising the article.

4.2 Serotonin homeostasis in the materno-foetal interface at term: Role of transporters (SERT/SLC6A4 and OCT3/SLC22A3) and monoamine oxidase A (MAO-A) in uptake and degradation of serotonin by human and rat term placenta

Karahoda R, Horackova H, Kastner P, Matthios P, Cerveny L, Kucera R, Kacerovsky M, Tebbens J, Bonnin A, Abad C, Staud F. *Acta Physiol (Oxf).* 2020;229(4):e13478. **(IF = 5.87, Q1)**

In this article, we describe the extensive investigation of placental serotonin handling, a crucial trophic factor for fetal development during pregnancy. Using *in situ* and *ex vivo* models of human and rat placenta, we characterized a novel physiological mechanism of massive serotonin extraction from the fetal circulation into the placenta by the organic cation transporter 3 (OCT3/SLC22A3). Contrary to current belief, we showed that both maternal- and placental-derived serotonin are metabolized by placental MAO-A; serotonin is transported across the term placenta to the fetus (regardless of origin) only if MAO-A is inhibited. We hypothesized that a synchronized activity of SERT, OCT3, and MAO-A is critical to protect the placenta and fetus from deleterious effects of excessive circulating serotonin.

Next, we used population-based mathematical modeling to characterize serotonin uptake from the fetal circulation. We reported an effect of fetal sex with male fetuses exhibiting different patterns of placental extraction and retention of serotonin compared to female ones. Additionally, we showed that serotonin uptake by OCT3 is inhibited by endogenous molecules (e.g. glucocorticoids) and exogenous agents (e.g. antidepressants) (Figure 7), suggesting that prenatal stress or exposure to these medications could alter this protective mechanism.

Based on these findings, we concluded that the placenta's basal (fetus-facing) membrane is essential in maintaining serotonin homeostasis in the fetal circulation. At the end of pregnancy, the placenta may play a protective role against toxic levels of serotonin in fetal circulation by taking it up into trophoblast cells (by OCT3/SERT transporters) and subsequent metabolism (by MAO-A) (Figure 7). Notably, the inhibition of placental OCT3 by pharmaceuticals opens a new window of potential, so far unforeseen, complications of medication use during pregnancy.

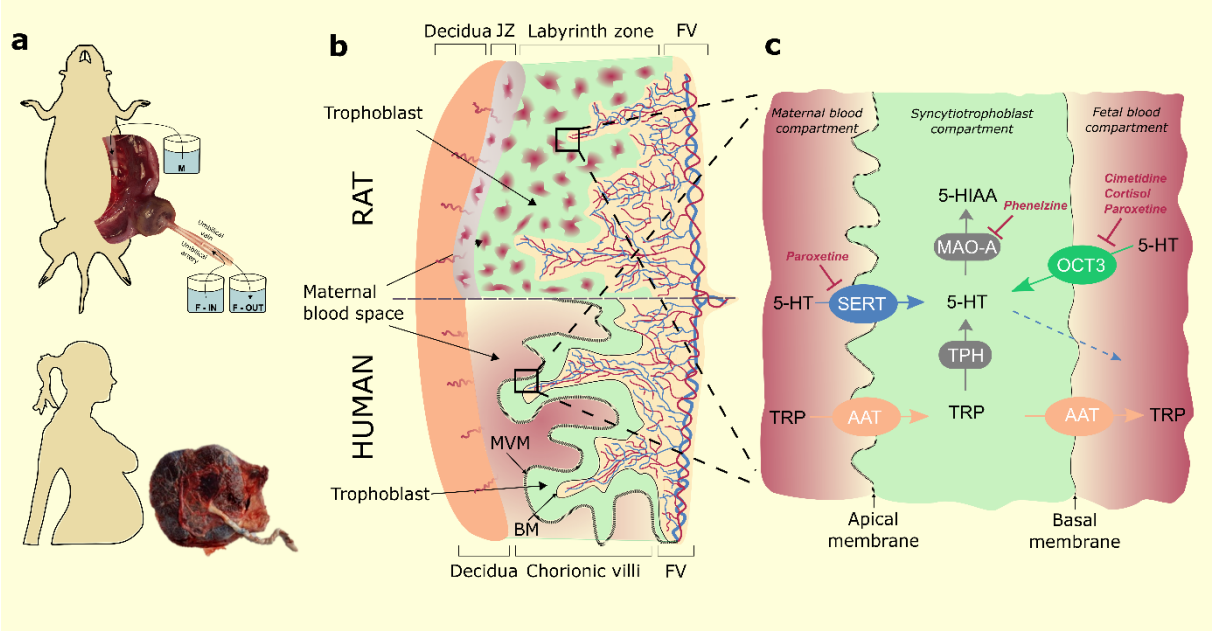

Figure 7. Graphical abstract depicting the main study findings. Experimental models (a) used in the study included both rat and human term placenta, which structurally (b) share the hemochorial arrangement; nonetheless, they differ in the type of maternal-fetal interdigitation (humans - villous type, rats - labyrinthine type). (c) Rat and human term placenta take up serotonin from maternal and fetal circulation via SERT- and OCT3-mediated uptake, respectively, for subsequent metabolism by MAO-A. These mechanisms are prone to inhibition by endogenous compounds and pharmacotherapy.

Abbreviations: 5-HT - serotonin, AAT - amino acid transporter, BM - basal membrane, F - fetal, FV - fetal vasculature, JZ - junctional zone, M - maternal, MAO-A - monoamine oxidase A, MVM - microvillous membrane, OCT3 - organic cation transporter 3, SERT - serotonin transporter, TRP - tryptophan.

Candidate's contribution:

- Performing experiments, specifically:
    - *In situ* dual perfusion of the rat term placenta
    - DNA isolation
    - Fetal sex determination by endpoint PCR analysis
    - Human placental sample collection
    - Isolation of plasma membranes from human term placenta and uptake studies
    - RNA isolation
    - Expression analysis by qPCR and ddPCR
    - Assistance in HPLC measurements
- Data analysis, interpretation of results, visualization
- Writing of the article and preparation for submission

4.3 Dynamics of Tryptophan Metabolic Pathways in Human Placenta and Placental-Derived Cells: Effect of Gestation Age and Trophoblast Differentiation

Karahoda R*, Abad C*, Horackova H, Kastner P, Zaugg J, Cerveny L, Kucera R, Albrecht C, Staud F. *Front Cell Dev Biol*. 2020;8:574034. **(IF = 5.201, Q - not available)**

In this article, we describe the prenatal dynamics of placental tryptophan metabolism along the serotonin and kynurenine pathways. It is a follow-up study to article 4.2, where we demonstrated a novel mechanism of serotonin uptake from the fetal circulation. Nevertheless, studies at earlier gestational ages have reported that placental metabolism of tryptophan to serotonin, and subsequent delivery to the fetal circulation, is crucial for embryonic brain development. Interestingly, the opposite appears to be true for tryptophan metabolism to kynurenine, which, according to the current literature, significantly increases at term. Thus, we hypothesized that the placental role in tryptophan utilization and serotonin handling changes during gestation. Additionally, we analyzed the effect of cell/trophoblast differentiation on gene expression patterns in isolated primary trophoblast cells and placenta-derived cell lines (BeWo, BeWo b30 clone, JEG-3) and assessed their suitability for placental tryptophan metabolism and transport studies.

We carried out a comprehensive investigation on the interplay between the two pathways during gestation. Specifically, we analyzed the gene expression of 16 enzymes and five transporters involved in the metabolism/transport of tryptophan and its metabolites in the human first trimester and term placenta. Subsequent protein expression analysis and functional enzymatic activity of the rate-limiting enzymes revealed preferential tryptophan utilization for serotonin and NAD^+ synthesis early in gestation. On the other hand, term placenta significantly produced kynurenine via IDO-mediated metabolism.

Notably, we showed that choriocarcinoma-derived cell lines do not share the same enzymatic and transport portfolio compared to primary trophoblast cells. Additionally, they show divergent and a more pronounced effect of differentiation, indicating that they are inadequate *in vitro* models for tryptophan-related placenta research. On the other hand, the gene expression of primary trophoblast cells resembled that of the human term placenta, thus designating them as the best cell-based model.

Collectively, we revealed that placental tryptophan homeostasis is subject to strictly regulated developmental changes, and fine-tuning of tryptophan along the serotonin or kynurenine

pathways is likely critical to ensure proper wiring between the placenta-brain axis (Figure 8). Importantly, both serotonin and kynurenine pathways are affected by insults such as disease, pharmacotherapy, and polymorphisms. Since the timing of insult also plays a critical role in fetal development, our results contribute to deciphering gestation-age dependent biological roots of fetal programming.

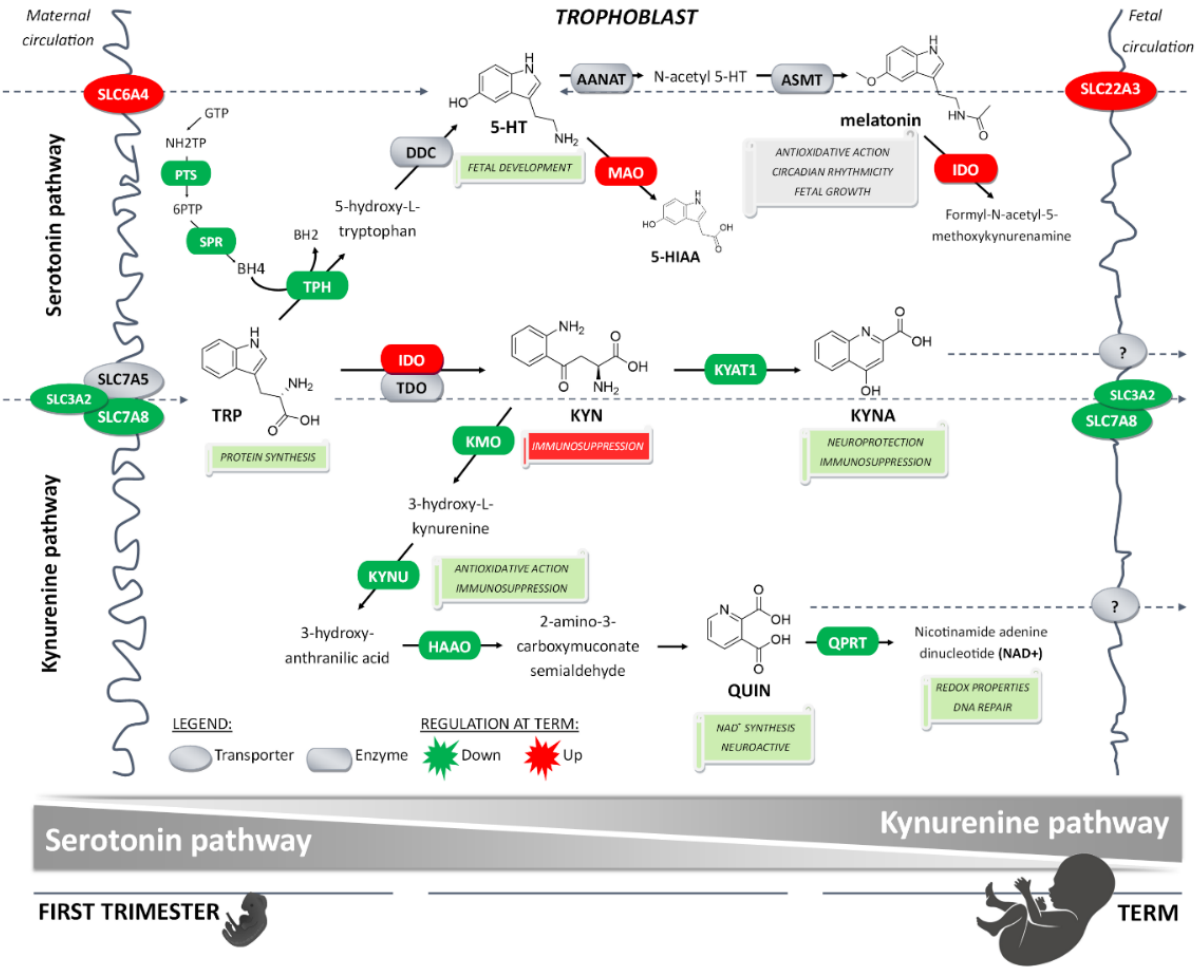

Figure 8. Graphical abstract depicting the main study findings. A fine-tuning of tryptophan metabolism in the human placenta occurs during gestation, with preferential serotonin synthesis early in pregnancy and a shift to the kynurenine pathway at term.

Abbreviations: 5-HT - serotonin, 5-OH-TRP - 5-hydroxytryptophan, 6PTP - 6-pyruvoyl-tetrahydrobiopterin, AANAT - aralkylamine N-acetyltransferase, ASMT - acetylserotonin O-methyltransferase, BH4 - tetrahydrobiopterin, GTP - guanosine triphosphate, HAAO - 3-hydroxyanthranilate 3,4-dioxygenase, IDO - indoleamine 2,3-dioxygenase, KMO - kynurenine 3-monooxygenase, KYAT1 - kynurenine aminotransferase 1, KYN - kynurenine, KYNA - kynurenic acid, KYNU - kynureninase, MAO - monoamine oxidase, NH2TP - 7,8-dihydroneopterin triphosphate, PTS - 6 pyruvoyltetrahydropterin synthase, QPRT - quinolinate phosphoribosyltransferase, QUIN - quinolinic acid, SPR - sepiapterin reductase, TDO - tryptophan 2,3-dioxygenase, TPH - tryptophan hydroxylase, TRP - tryptophan

Candidate's contribution:

- Performing experiments, specifically:
    - Cell culture and treatment
    - RNA isolation
    - Human placental sample collection
    - Expression analysis by qPCR and ddPCR
    - Preparation of placental homogenates
    - Functional analysis of enzymes
- Data analysis, interpretation of results, visualization
- Writing of article and preparation for submission

**The authors contributed equally to this work.*

4.4 Profiling of Tryptophan Metabolic Pathways in the Rat Fetoplacental Unit During Gestation

Abad C*, **Karahoda R***, Kastner P, Portillo R, Horackova H, Kucera R, Nachtigal P, Staud F. *Int J Mol Sci.* 2020;21(20). **(IF = 4.556, Q2)**

In this article, we characterize tryptophan metabolism along the serotonin and kynurenine pathways in the rat placenta and fetal organs during gestation. In article 4.3, focused on the human placenta, we have shown that a tight regulation exists in the expression and/or activity of placental enzymes and transporters directly or indirectly involved in tryptophan metabolic pathways. In this study, we hypothesized that apart from the placenta, fetal organs also contribute to overall tryptophan homeostasis in the fetoplacental unit. However, experiments in pregnant women are complicated due to ethical and technical reasons, and investigating fetal organs is impossible. Therefore, here we used the Wistar rat, suggested as the most appropriate alternative model for placental tryptophan metabolism in health and disease. We provide detailed insights into prenatal dynamics of tryptophan metabolism not only in the placenta but also in fetal organs during gestation.

Employing gene and protein expression analyses and functional enzymatic activity studies, we showed for the first time that, in concord with our hypothesis, tryptophan is preferentially utilized by the placenta for serotonin synthesis early in gestation. On the other hand, a decrease in placental serotonin synthesis towards the end of gestation reflects the fact, that the fetus can synthesize its own serotonin from maternal tryptophan at term. In contrast, placental kynurenine production increased with gestation, and fetal organs showed minimal production in the prenatal period.

Collectively, we demonstrated that placental dynamics of both serotonin and kynurenine pathways are primarily driven by the demands of the developing fetus (Figure 9). Importantly, our data obtained from the rat placenta are in close agreement with those observed in humans (article 4.3), confirming the Wistar rat as an appropriate model for further studies on tryptophan homeostasis in the fetoplacental unit.

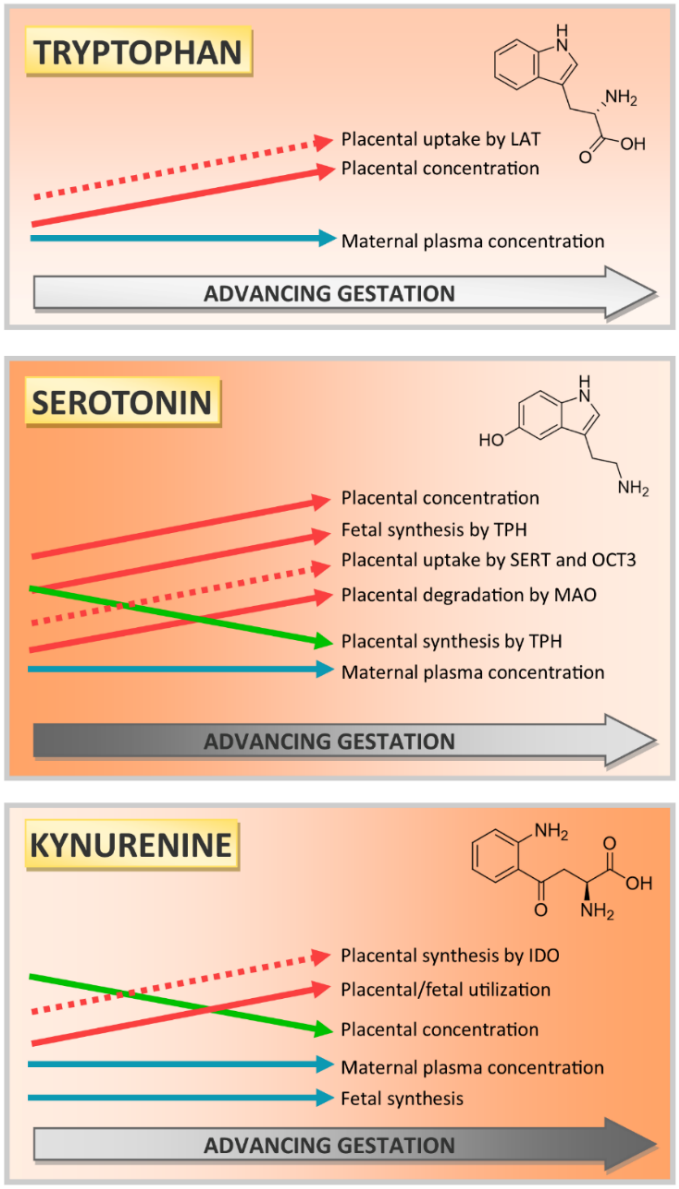

Figure 9. Graphical abstract depicting the main study findings. Placental tryptophan metabolism changes throughout gestation to reflect fetal demands for serotonin and kynurenine metabolites.

Abbreviations: IDO - indoleamine 2,3-dioxygenase, LAT - L type amino acid transporter, MAO - monoamine oxidase, OCT3 - organic cation transporter 3, SERT - serotonin transporter, TPH - tryptophan hydroxylase.

Candidate's contribution:

- Performing experiments, specifically:
    - Rat placental and fetal sample collection
    - RNA isolation
    - Expression analysis by qPCR and ddPCR
    - Preparation of organ homogenates
    - Functional analysis of enzymes
- Data analysis, interpretation of results, visualization
- Writing of the article and preparation for submission

**The authors contributed equally to this work.*

4.5 Revisiting the molecular targets of serotonin reuptake inhibitors in the fetoplacental unit: maternal and fetal perspective

Horackova H, **Karahoda R**, Cerveny L, Vachalova V, Ebner R, Abad C, Staud F. *Submitted (January 2021)*

Nowadays, up to 13% of pregnant women are prescribed antidepressants, despite their negative impact on pregnancy outcomes. In this article, we investigated six antidepressants and their effect on serotonin homeostasis in the placenta. In article 4.2, we have described the importance of two membrane transporters for placental uptake of serotonin: SERT, localized in the placenta's apical, mother-facing membrane, and OCT3, localized in its basal, fetus-facing membrane. Since currently used antidepressants can inhibit both SERT and OCT3, we investigated their inhibitory effects on these transporters using *in situ* and *ex vivo* models of human and rat placenta.

Notably, we found that paroxetine was the most potent inhibitor of both SERT and OCT3, and the strongest disruptor of placental serotonin homeostasis. Interestingly, paroxetine is the antidepressant most frequently associated with poor fetal development, including increased risks of septal heart defects, cardiovascular malformations, and neonatal withdrawal symptoms. We hypothesized that this inhibition leads to critical serotonin accumulation in both maternal and fetal circulations and contributes to the detrimental consequences of depression treatment during gestation. Besides, we detected an apparent effect of fetal sex, as antidepressants' inhibition of OCT3 in rat placenta was stronger when fetuses were male. This is in line with higher reported risks of neurological disorders after prenatal use of antidepressants for males. Our data also showed that this association was independent of OCT3 transcript and protein levels and both placental MAOA activity and placental lipid peroxidation.

Lastly, we carried out *in vitro* experiments employing MDCKII cells (transfected with P-gp, BCRP and MRP2 efflux transporters) and *in situ* dually perfused rat term placenta to assay potential interaction between the tested antidepressants and placental efflux transporters. We did not reveal any significant interaction between the tested antidepressants and placental efflux transporters.

Collectively, we provided novel mechanisms of antidepressants' effects on placental serotonin homeostasis. Our results indicated that even half-maximal inhibitory concentrations might be reached in the fetal circulation. We thus speculate that the reported mechanisms likely

contribute to associated changes in fetal development and poorly reported outcomes of antidepressant use during gestation.

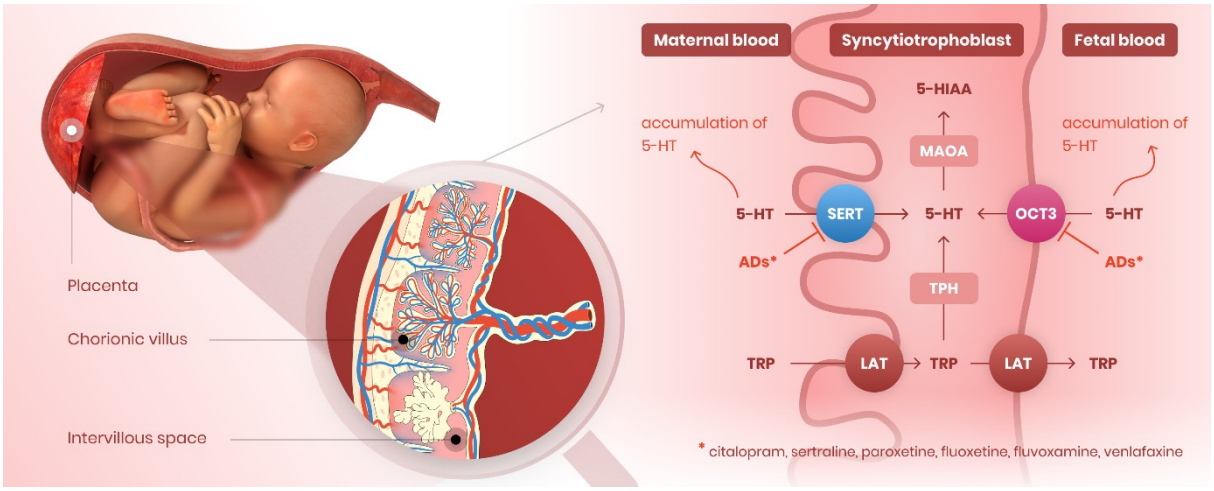

Figure 10. Graphical abstract depicting the main study findings. Antidepressant drugs (paroxetine, citalopram, venlafaxine, fluoxetine, fluvoxamine, and sertraline) inhibit placental SERT- and OCT3-mediated serotonin uptake and thus disturb placental serotonin homeostasis.

Abbreviations: 5-HT - serotonin, ADs - antidepressants, LAT - L type amino acid transporter, MAOA - monoamine oxidase A, OCT3 - organic cation transporter 3, SERT - serotonin transporter, TPH - tryptophan hydroxylase, TRP - tryptophan.

Candidate's contribution:

- Performing experiments, specifically:
  - *In situ* dual perfusion of the rat term placenta
  - Human placental sample collection
  - Isolation of plasma membranes from human term placenta
- Assisted in data analysis, interpretation of results, visualization
- Assisted in writing the article and preparation for submission

5 SUMMARY

Pregnancy is a dynamic state undergoing continuous physiological changes in order to meet placental and fetal requirements for growth and development. Latest research highlights the paramount importance of the crosstalk between the placenta and fetal organs, as a mutual communication and collaboration, for proper *in utero* development and fetal programming [134]. Moreover, the influence of prenatal insults on placental functions is now considered as one of the main mechanisms contributing to adulthood diseases [7]. In this thesis, we provide a comprehensive characterization of tryptophan metabolism in the fetoplacental unit during gestation. Further, we investigate the potential of pharmacotherapy in pregnancy (specifically antidepressant drugs) to interfere with the placental homeostasis of serotonin.

During pregnancy, the needs for the essential amino acid tryptophan increase [8]. Tryptophan delivery to the fetus is achieved through transport from maternal circulation via LAT1 (SLC7A5) on the maternal-facing membrane and LAT2 (SLC7A8) on both maternal- and fetal-facing membranes [135]. In line with increasing tryptophan demand, we observed that placental tryptophan levels and the expression of *Slc7a5* and *Slc7a8* increase with advancing gestation in rats. We propose that these changes are critical to ensure tryptophan availability for protein synthesis and for the generation of neurotransmitters, hormones, and other bioactive molecules.

In mammals, the kynurenine pathway represents the major tryptophan catabolic route in many tissues, including the placenta [8]. IDO1 catalyzes the rate-limiting step of tryptophan metabolism along the kynurenine pathway. We and others [95, 96] reported IDO to be modestly expressed in the first-trimester human placenta and upregulated at term. On the contrary, we demonstrated that the first-trimester placentas show preferential expression of downstream kynurenine pathway enzymes involved in the generation of KYNA and QUIN. Nonetheless, while IDO expression and activity is minimal at this time in pregnancy, TDO (an enzyme closely related to IDO) is stably expressed throughout gestation. Our results support a notion proposed by Badawy [8] in which tryptophan degradation in early-to-mid pregnancy is catalyzed by TDO, with IDO gaining a partial/transient role in mid-gestation.

Subsequent experiments in rats revealed that the rat placenta and fetal organs (brain, intestine, liver, and lungs) do not express the Ido1 gene; instead, Ido2 is the predominant isoform. Its functional activity remained unchanged from mid-gestation to term in rats. Nevertheless, using immunohistochemical staining, we reported that its protein localization in the vascular endothelium coincides with IDO1 in the human placenta [96]. Interestingly, the placental

content of kynurenine in rats decreased significantly towards term. To evaluate whether this is due to kynurenine transport to the fetal circulation, we investigated IDO expression and activity in fetal organs at term. While the fetal liver showed the highest Ido2 transcripts, its activity was notably lower, with the placenta exhibiting the most pronounced IDO activity. This was also previously reported for TDO, where absent activity was observed in the liver of fetuses and young rats [136, 137]. These findings collectively suggest that fetal organs are not yet fully functional for kynurenine production, and placental kynurenine synthesis and transport appear to be the principal fetal source throughout gestation.

Altogether, we speculate that, in the first trimester, tryptophan metabolism to kynurenine via TDO serves mainly as a precursor of kynurenine metabolites, including KYNA and QUIN. These metabolites are essential in NAD^+ synthesis, redox reactions, DNA repair and exhibit antioxidant and immunosuppressive properties [8]. On the other hand, the significant increase in IDO1 at term could account for high kynurenine production involved in the immune-related activities. This concept was pioneered by Badawy, suggesting preferential tryptophan utilization for protein, serotonin, and NAD^+ synthesis in early pregnancy [8].

Serotonin is an important neurotransmitter derived from tryptophan and its concentrations within the fetoplacental units must be tightly regulated for adequate development. Currently, the placenta has been regarded as the organ, that to a certain extent controls serotonin levels in the fetoplacental unit [138]. Nonetheless, research on placental serotonin handling has been controversial, with some studies showing transfer from maternal circulation [108], whereas others indicating serotonin synthesis within the placenta [55, 91]. To investigate maternal-to-fetal transport and/or placental synthesis of serotonin, we performed *in situ* perfusion of rat term placenta, infusing the maternal side with serotonin or tryptophan and quantifying the concentrations of tryptophan, serotonin, and its metabolites in the fetal circulation. We showed that there is negligible maternal-to-fetal transport of serotonin at term under basal conditions, consistent with data in mice [55].

Interestingly, when placental MAO-A was inhibited using phenelzine, we observed placental serotonin release into the fetal circulation, indicative of residual neosynthetic and transport capacity in term placentas. Altogether, this suggests that in contrast to early pregnancy, the term placenta highly metabolizes serotonin and no longer transfers maternal or placenta-synthesized serotonin to the fetus. We thus hypothesized that placental handling of serotonin changes during gestation. While early in gestation, the fetus is not capable of serotonin synthesis, the placenta

serves as a transient serotonin source [55, 91, 108]. On the other hand, at term, once the fetus is capable of serotonin synthesis [110, 111], the placenta chiefly controls its levels via the activity of MAO-A [139].

To address this issue, we carried out expression and functional analysis of tryptophan pathways in human (first trimester vs. term) and rat (gestational day 12, 15, 18, and 21) placenta during gestation. In rats, we observed an increase in placental levels of serotonin, despite steady concentrations in maternal blood, a phenomenon previously reported by Robson and Senior [107]. To investigate whether the rise in placenta concentrations may be due to placental serotonin synthesis, we analyzed the expression and activity of TPH, the rate-limiting component responsible for serotonin synthesis. We further investigated the placental content of 5-hydroxytryptophan (5-OH-TRP), an intermediate metabolite in serotonin production from tryptophan. We observed decreased placental 5-OH-TRP concentration and 5-OH-TRP/TRP ratio during gestation, which indicated decreased placental serotonin synthesis towards term. We also reported lower *Tph1* transcripts and TPH protein at the final stages of rat pregnancy. Moreover, the expression of 6-pyruvoyltetrahydropterin synthase (PTS) and sepiapterin reductase (SPR), necessary for the synthesis of BH4, decreased significantly in the term placenta. With BH4 serving as a cofactor for endothelial nitric oxide synthase, we speculate that decreasing SPR expression and activity [140] at term may decrease the availability of BH4 for TPH activity, thus serotonin synthesis. These results collectively indicate that placental tryptophan metabolism to serotonin is more pronounced at the beginning of pregnancy, with the neosynthetic capacity decreasing at term.

Indeed, it has been shown that placental serotonin synthesis occurs as early as E10.5 in mice and week 11 in humans [55], a period during which the fetus is not capable of its own serotonin synthesis [57]. With decreased placental serotonin supply at term, we next ought to determine the fetal serotonin synthetic capacity in late gestation. Fetal intestine, brain, lungs, and liver at gestation day 18 and 21 were investigated for Tph expression and activity. All organs evaluated showed Tph1 transcripts higher than those of the term placenta; however, only the fetal brain and intestine showed functional TPH activity. These findings correspond with previously published research reporting utilization of maternal tryptophan for serotonin synthesis by the rat fetuses at term [110, 111].

Nonetheless, considering the fetal serotonin-synthesizing capacity in late gestation, it is surprising that no attempt was made to investigate placental handling of serotonin

(secretion/extraction) on the basal, fetus-facing membrane of the placenta. Apart from SERT, serotonin is a substrate of several organic cation transporters [66] and plasma membrane monoamine transporter (PMAT, *SLC29A4*) [141]. Of these, only OCT3 (*SLC22A3*) is abundant in the basal membrane of syncytiotrophoblast [72] and has been previously shown to extract neurotoxins from the fetal circulation [74]. Thus, we hypothesized that placental OCT3 might facilitate the extraction of serotonin from the fetal circulation at term. Using a set of experimental approaches in rat and human placenta, we provided the first evidence that the placenta massively extracts serotonin from the fetal circulation into trophoblast cells. This uptake was mediated by the high-capacity OCT3 in a concentration-dependent manner and was inhibitable by endogenous (glucocorticoids) and exogenous (pharmaceuticals) agents. This observation opened new windows to investigate previously unsuspected insults during pregnancy such as prenatal glucocorticoid excess or medication of pregnant women with OCT3 inhibitors.

Notably, we observed considerable interindividual variability in placental extraction of fetal serotonin in rat. Thus, we employed population-based analysis to evaluate the effects of multiple factors as potential covariates and identified fetal sex as a factor influencing the transporter-mediated kinetics. While this effect was not attributable to differences in OCT3 transcripts, it may at least partly explain sex-dependent effects observed in behavioral studies of prenatal exposure to OCT3 inhibitors, such as metformin [142] or antidepressants [143].

Importantly, OCT3 expression and activity in the human placenta [141], rat placenta, and fetal brain [144] were previously shown to be upregulated towards the end of gestation, indicating the increasing importance of this transporter throughout gestation. We hypothesized that at term, orchestration between SERT, OCT3, and MAO-A serves as a serotonin detoxification mechanism, protecting the fetoplacental unit from high serotonin levels. Therefore, we investigated their expression and activity in human and rat placenta during gestation. In both models, we observed synchronized upregulation of transporters at term, which we conclude to be the mechanism behind increased placental serotonin levels in the rat term placenta. In line with previous reports [114, 115, 145], we showed that MAO-A is up-regulated in the final phases of pregnancy; thus, the extracted serotonin is efficiently degraded to inactive 5-HIAA. Furthermore, in the rat term placenta, we reported co-localization of SERT, OCT3 [74], and MAO in syncytiotrophoblast cells, specifically, layer II and III within the labyrinth area. These findings support the hypothesis of a placental serotonin clearance system, in which SERT, OCT3, and MAO-A seem to be the key components.

To investigate whether fetal mechanisms can control circulating serotonin levels, we also analyzed the expression and activity of MAO in the fetal organs and compared them with those of the placenta. We observed that the highest MAO activity in the prenatal period comes from the placenta and fetal brain, followed by the intestine, lungs, and liver. These findings agree with previous reports showing predominant MAO activity in the placenta [146, 147]. Consequently, proper placenta-brain axis wiring appears fundamental for regulating serotonin circulating levels in the fetoplacental unit and ensuring proper neurodevelopment of the fetus [134].

Altogether, our data suggest OCT3 as an essential component of serotonin homeostasis in the fetoplacental unit, and we propose that genetic, endocrine, or pharmacological insults of OCT3 expression/function may perturb placental serotonin handling and hence fetal development and programming. Notably, the potential of selected antidepressants to inhibit OCT3 function has been recently reported [148]. Nonetheless, the relevance of this interaction in the placental barrier has not been investigated to date. Since antidepressants inhibit both SERT and OCT3, we hypothesized that they might interfere with prenatal serotonin homeostasis by affecting its placental clearance on both maternal and fetal sides of the placenta. We investigated six serotonin reuptake inhibitors (SRIs) most frequently used in pregnancy (paroxetine, citalopram, fluoxetine, fluvoxamine, sertraline, and venlafaxine) using membrane vesicles isolated from human term placentas and *in situ* perfused rat term placenta. We presented the first evidence that in addition to SERT, antidepressants affect the function of placental OCT3. Importantly, the calculated IC_{50} values were in the range of therapeutically reachable plasma concentrations. Interestingly, male placentas were more sensitive to the inhibitory effect of SRIs, independent of OCT3 protein expression, placental MAO activity, or lipid peroxidation. We speculate that this mechanisms may partly explain the fetal sex-dependent variations observed in behavioral studies and increased risks of neurodevelopmental disorders upon prenatal treatment with antidepressants in males [149, 150].

In summary, our findings indicate novel mechanisms whereby SRIs reach fetal circulation and at therapeutic levels may affect the fetoplacental homeostasis of serotonin and contribute to poor pregnancy outcomes. We suggest that this effect can result in suboptimal serotonin concentrations in the fetoplacental unit, thereby jeopardizing fetal development and/or programming.

6 CONCLUSIONS

In conclusion, we report that the placental homeostasis of tryptophan is a complex network of numerous genes and subject to strictly controlled developmental changes during pregnancy. Considering the various roles of tryptophan and its metabolites in placenta function, fetal development, and programming, tight regulation is necessary to maintain endocrine homeostasis in the fetoplacental unit. Subsequently, internal or external insults, including pharmaceuticals, pathological conditions, environmental factors, polymorphisms and/or epigenetics, may compromise this harmonized interplay of enzymes and transporters, resulting in suboptimal *in utero* conditions. Notably, the time-frame of pregnancy during which these insults occur is critical for fetal development [151]. Therefore, detailed knowledge of the tryptophan catabolic pathways in the placenta is critical to understand the biological roots of fetal programming. Importantly, our data obtained from the rat placenta are in line with those observed in humans, confirming the Wistar rat as an appropriate animal model for studies on tryptophan homeostasis in the fetoplacental unit.

Further, our results demonstrate that OCT3 is a crucial component regulating fetoplacental homeostasis of serotonin. As a polyspecific transport system, it can be blocked by numerous molecules of endogenous (glucocorticoids) or exogenous (pharmaceuticals) origin. As various mechanisms of glucocorticoid-serotonin interactions have been described in the CNS [152, 153] and other organs [154], our results reveal possible interactions between these hormones in the fetoplacental unit. In addition, pharmaceuticals are often used during pregnancy despite the lack of safety data. We show that antidepressants are potent inhibitors of not only SERT but also OCT3 in human and rat placenta. Blocking SERT- and OCT3-mediated protective mechanism could potentially expose the fetoplacental unit to elevated serotonin concentrations and jeopardize serotonin-dependent neurogenic and other developmental processes. Importantly, prenatal use of other OCT3 inhibitors, such as metformin for gestational diabetes mellitus [142] or antiretrovirals for HIV positive pregnant women [155] might also dysregulate placental handling of serotonin and contribute to poor pregnancy outcomes. Collectively, our findings provide new mechanistic understandings of unforeseen complications during pregnancy, including prenatal glucocorticoid excess and/or pharmacotherapy use by pregnant women.

7 LIST OF OTHER OUTPUTS OF THE CANDIDATE

7.1 Original articles unrelated to the topic of the dissertation

- **Karahoda R**, Robles M, Abad C, Marushka J, Stranik J, Horackova H, Tebbens J, Vaillancourt C, Kacerovsky M, Staud F. Placental expression signature of tryptophan metabolism associated with term and spontanenous preterm birth. *Submitted (February 2021)*
- **Karahoda R,** Kallol S, Groessl M, Ontsouka E, Anderle P, Flueck C, Staud F, Albrecht C. Revisiting the steroidogenic pathways in human placenta and primary human trophoblast cells. *In press. Int J Mol Sci.* **(IF = 4.556, Q2)**
- Karbanova S, Cerveny L, Jiraskova L, **Karahoda R**, Ceckova M, Ptackova Z, Staud F. Transport of ribavirin across the rat and human placental barrier: roles of nucleoside and ATP-binding cassette drug efflux transporters. *Biochem Pharmacol*. 2019;163:60-70. **(IF = 4.24, Q1)**
- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of antiretroviral drugs on the L-carnitine uptake in human placenta. *Toxicol Appl Pharmacol*. 2019;368:18-25. **(IF = 3.616, Q1)**
- Cerveny L, Ptackova Z, Ceckova M, **Karahoda R**, Karbanova S, Jiraskova L, Greenwood SL, Glazier JD, Staud F. Equilibrative Nucleoside Transporter 1 (ENT1) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta. *Drug Metab Dispos*. 2018;46(11):1817-1826. **(IF = 3.64, Q1)**

7.2 Oral presentations related to the topic of the dissertation

- **Karahoda R**, Abad C, Staud F. Prenatal dynamics of tryptophan metabolism; A study on human and rat placenta. *13th European Placental Perfusion Workshop* (2020) – Virtual
- **Karahoda R**, Staud F. Placental homeostasis of tryptophan and monoamines in health and disease. *Placenta Interface Seminar Series* (2020) - Virtual
- **Karahoda R**, Horackova H, Cerveny L, Abad C, Staud F. Sex-dependent differences in placental serotonin handling; Organic cation transporter 3 (OCT3/SLC22A3) - A new piece of the placental serotonin puzzle. *IFPA Conference; Placenta: the origin of pregnancy health and disease* (2019) – Buenos Aires, AR
- **Karahoda R**, Horackova H, Cerveny L, Abad C, Staud F. Organic cation transporter 3 (OCT3/SLC22A3) – a new piece of the placental serotonin puzzle. *12th European Placental Perfusion Workshop* (2019) – Nijmegen, NL

- **Karahoda R**, Kastner P, Horackova H, Cerveny L, Kucera R, Abad C, Staud F. Placental transport and metabolism of serotonin and tryptophan. *9th Postgradual and 7th Postdoctoral Scientific Conference* (2019) – Hradec Králové, CZ
- **Karahoda R**, Cerveny L, Kastner P, Kucera R, Staud F. Expression and function of transporters/enzymes involved in placental metabolism of tryptophan. *68th Czech-Slovak Pharmacological Days* (2018) – Hradec Králové, CZ

7.3 Poster/oral presentations unrelated to the topic of the dissertation

- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of antiretroviral drugs on the transport of L-carnitine in human placenta. *8th Postgradual and 6th Postdoctoral Scientific Conference* (2018) – Hradec Králové, CZ
- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of anti-hepatitis C drugs on the transport of L-carnitine in human placenta. IFPA Conference; Clinical Growth via Placenta (2018) – Tokyo, JP
- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of antiretroviral drugs on the transport of L-carnitine in human placenta. *11th European Placental Perfusion Workshop* (2018) – Hradec Králové, CZ
- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of antiretroviral drugs on the transport of L-carnitine in human placenta. Meet the Experts Transporter Conference (2018) – Budapest, HU
- **Karahoda R**, Ceckova M, Staud F. The inhibitory effect of antiretroviral drugs on L-carnitine transport in the placenta. SFB35 Transmembrane Transporters in Health and Disease (2017) – Vienna, AT

7.4 Grant projects

Principal investigator

- Rector's Mobility Fund; 2019; Grant number: FM/c/2019-1-093
- Grant Agency of Charles University; 2017-2019; Grant number: 1574217/C/2017; Title of project: *Study on interaction of antiretroviral drugs with uptake transporters expressed in the placental microvillous membrane*

Team member

- Czech Science Foundation; 2020-2023; Grant number: 20-13017S; Title of project: *Antidepressants in pregnancy; effect on placental transport and metabolism of serotonin*

- Czech Health Research Council; 2020-2024; Grant number: NU20-01-00264; Title of project: *Placental tryptophan metabolism linking maternal inflammation and foetal neurodevelopmental disorders*
- Grant Agency of Charles University; 2019-2021; Grant number: 1464119/C/2019; Title of project: *Antidepressants in pregnancy; mother-to-fetus transport and effect on placental transport and metabolism of serotonin*
- Czech Science Foundation; 2017-2019; Grant number: GACR 17-16169S; Title of project: *In vitro, in situ and ex vivo study of interactions of novel antiviral agents with drug transporters; effect on their passage across the placenta*

7.5 Scientific experience abroad

- 6-month laboratory training at the Institute of Biochemistry and Molecular Biology, University of Bern (Prof. Christiane Albrecht), Switzerland; 2019/20
- 1-week laboratory training at Placenta Lab, Jena University Hospital (Prof. Udo Markert), Germany; 2019

7.6 Awards and scholarships attained during the studies

- 1st place at Angelini University Award (team competition) - Angelini Pharma Česká republika - September 2020
- YW Loke New Investigator Travel Award - International Federation of Placenta Associations - September 2019
- Doctoral student scholarship - Ministry of Education, Science and Technology, Republic of Kosovo - January 2017

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