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ENDOCANNABINOID CROSSTALK BETWEEN PLACENTA AND MATERNAL FAT IN A BABOON MODEL (*Papio spp.*) OF OBESITY

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Abstract

Introduction—Maternal obesity (MO) remains a serious obstetric problem with acute and chronic morbidities for both mothers and offspring. The mechanisms underlying these adverse consequences of MO remain unknown. Endocannabinoids (ECB) are neuromodulatory lipids derived from adipocytes. Metabolic crosstalk between placenta and adipocytes may mediate sequelae of MO. The goal of this study was to elucidate placental and systemic ECBs in MO.

Material and methods—Placentas, serum, and subcutaneous fat were collected at Cesarean sections performed near term (0.9 G) in four non-obese (nOB) and four obese (OB) baboons (*Papio spp.*). Concentrations of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were measured by liquid chromatography coupled to tandem mass spectrometry. AEA and 2-AG pathways were characterized in placentas by Q-RT-PCR, Western blot, and immunohistochemistry.

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Results—Placental 2-AG levels were lower and maternal fat AEA levels were higher in OB (1254.1 ± 401.3 nmol/kg and 17.3 ± 4 nmol/kg) vs. nOB (3124.2 ± 557.3 nmol/kg and 3.1 ± 0.6 nmol/kg) animals. Concentrations of 2-AG correlated positively between maternal fat and placenta ($r=0.82$, $p=0.013$), but correlated negatively with maternal leptin concentrations ($r=-0.72$, $p=0.04$ and $r=-0.83$, $p=0.01$, respectively).

Conclusion—This is the first study to demonstrate differential ECB pathway regulation in maternal fat and placenta in MO. Differential regulation and function exist for AEA and 2-AG as the major ECB pathways in placenta.

INTRODUCTION

Maternal obesity (MO) remains at epidemic dimensions in developed countries [1, 2] and exerts adverse consequences across the lifespan upon the mother, her fetus, the newborn, and later adult life [3]. Mechanisms linking MO and negative pregnancy outcomes remain unknown. Activation of the endocannabinoid system (ECBs) has been reported as a consequence of obesity [4, 5] and been associated with higher endocannabinoid (ECB) levels in visceral fat and serum from obese subjects [6, 7].

The ECB system includes N-arachidonylethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG), cannabinoid receptors 1 and 2 (CB1 and CB2), the recently added orphan receptor, GPR55, and specified enzymes for their synthesis and degradation [8, 9]. AEA biosynthesis occurs via cleavage of N-arachidonoylphosphatidylethanolamine (NAPE) by the ECB-specific enzyme NAPE-phospholipase D (NAPE-PLD). Combined actions of phospholipase C and diacylglycerol lipase (DAGL) produce 2-AG. [10]. Degradation of AEA is mediated by fatty acid amide hydrolase (FAAH), which hydrolyzes the amide bond to yield arachidonic acid (AA) and ethanolamine. The enzyme monoacylglycerol lipase (MAGL) is the main catabolic enzyme producing AA and glycerol, however FAAH, -hydrolase 6 (ABHD6) and 12 (ABHD12) can also catabolize 2-AG [9–12].

CB1 receptors are abundant in the central nervous system but also detected in peripheral organs, including adrenal gland, heart, uterus, gonads, and liver [13, 14]. CB2 receptors have been identified mainly in the central nervous system on microglia besides peripheral occurrence [15, 16]. ECBs and ECB signaling are emerging as key regulators of reproduction, as evidenced by tight regulation of uterine ECB signaling and their critical roles in preimplantation embryo development, oviductal embryo transport, implantation, and placentation [17, 18]. Moreover, the human fetus may depend upon placental and maternal sources of ECBs [19]. We previously described that placental, fetal, and maternal responses to MO in baboons (*Papio spp.*) strikingly resemble the changes described in human pregnancies [20, 21]. The present work aimed to characterize ECB responses to MO in a baboon model, based on hypothesis that local (placental and maternal fat) and circulating ECB tone would increase in obese mothers.

MATERIAL AND METHODS

Animals housing and handling

Animals' characteristics and procedures were previously described in details [20]. Briefly, animals housed in harem cages at the AALAC-approved facilities of the Southwest National Primate Center at the Texas Biomedical Research Institute were divided into two groups [Obese (Ob) $n=4$ (two male and two female fetuses), and non-Obese (nOb) $n=4$ (all fetuses are males)] based on the Rh index. Rh index is an obesity index defined as body weight divided by the square of the crown-rump length in another non-human primate species

(Rhesus monkeys). Rh indices were $48.7 \pm 1.0 \text{ kg/m}^2$ for Ob and $39.1 \pm 3.2 \text{ kg/m}^2$ for nOb animals [20]. All procedures were approved by the Animal Care and Use Committee of the Texas Biomedical Research Institute.

Tissue collection and processing

Farley *et al.*, 2009 detailed the Cesarean procedures performed at the end of gestation [165 days of gestation (dGA, term = 180 dGA)], maternal and fetal tissue collections, and organ weighing [20]. Maternal subcutaneous fat tissue was collected under the umbilicus during necropsy. Maternal and fetal organ weights are presented on Table 1. Subcutaneous fat and placental tissues were flash-frozen in liquid nitrogen and stored at -80°C until further use. Additionally, a sample from the central part of the placenta was embedded in paraffin, cut at 5 microns and processed as described below.

Measurements of circulating and peripheral hormones

Leptin—Circulating leptin was measured by radioimmunoassay in a single assay according to the manufacturer's instructions (LINCO Research, Inc., St. Charles, MO) as described previously. Intra-assay coefficient of variation (%CV) was 3.0% at leptin concentration of 4.9 ng/mL [21].

AEA and 2AG—AEA and 2-AG concentrations were determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as previously described [22]. Briefly, 1 mL serum was spiked with the internal standards (IS) d_4 -AEA and d_5 -2-AG to a final concentration of 1.2 nM and 1.8 nM, respectively, immediately after thawing on ice. Liquid-liquid extraction was performed with 1 mL toluene. For placental tissue, the IS (final concentration of 12.5 nmol/kg d_4 -AEA and 3.8 nM/kg d_5 -2-AG) was added to ≈ 100 mg deep-frozen tissue. One-step homogenization and liquid-liquid extraction with toluene was performed by means of a cooled Precellys 24 homogenizer (Bertin Technologies, France). Maternal fat tissue (≈ 100 mg) was spiked with a fixed volume of IS solution resulting in varying concentrations (≈ 30 – 40 nmol/kg d_4 -AEA and 9–14 nmol/kg d_5 -2-AG). Solid phase extraction of ECBs was performed after homogenization of fat tissue, IS, and 1 mL chloroform with a Precellys 24 homogenizer.

RT-PCR

Total RNA was extracted from 70 mg of homogenized placental tissues using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), cDNA was synthesized with first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany) from 5 μg of total RNA following the manufacturer's instructions as previously described. Quantitative PCR was performed in triplicates with custom-synthesized (Thermo Fisher Scientific Inc, Waltham, MA, USA) and commercially available (Roche, Applied sciences Indianapolis, IN, USA) sequence-specific oligonucleotides (Table 2A), using TaqMan Mix (Roche, Applied sciences Indianapolis, IN, USA) or SYBR green mix (Kapa Bio systems Inc, Woburn, MA, USA) (Table 2A) [23]. Data were collected on a LightCycler[®] 480 (Roche, Applied Sciences, Indianapolis, IN, USA). Relative mRNA expression data were calculated using the standard C_t method [23].

Immunohistochemistry

Deparaffinized (5 μm thick) baboon's placenta slides were placed in antigen retrieval solution (Citrate Buffer, pH=6) and pressure cooked for 30 minutes (FAAH antibodies), or microwaved twice for 2 min (for CB1 and CB2) or 4 min (other antibodies). The avidin-biotin complex (ABC) method was employed according to the manufacturer's guidelines (Vectastain ABC kit, Vector Laboratories, Inc. Burlingame, CA). Table 2 B lists primary

antibodies and dilutions used. Immunological complexes were visualized by the use of H₂O₂/DAB (3,3'-diaminobenzidine-tetrahydrochloride) as substrate/chromogen for CB1, FAAH, and NAPE-PLD; MAGL and H₂O₂/AEC (3-Amino-9-Ethylcarbazole) were used as substrate/chromogen for CB2 and DAGL-. Slides with FAAH immunostaining were counterstained with hematoxylin. Slides were mounted with Dako Mounting Medium (Dako, Carpinteria, CA).

Histomorphometry

Slides were scanned using an AperioScanScope® instrument at 40X. Images were analyzed using the ImageScope™ v11.1.2.752 by Aperio® available positive pixel count algorithm as described elsewhere [24]. One randomly selected slide from the central part of the placenta was used. Immunostaining levels in the villi (trophoblast, mesenchymal core, fetal capillaries) and the basal plates were obtained separately using histoscore method [25, 26].

Western Blot

To evaluate expression changes observed in *CB1* and *CB2* receptors, Western blot (WB) was performed with suitable antibodies (Table 1B), as described elsewhere [23]. Protein expression was quantified using arbitrary units (optical density of the bands relative to the beta-actin expression) with Image J (NIH) program [23].

Statistical methods

Comparisons between obese and non-obese groups were performed by two-tailed Student's t-tests. Correlation of variables was performed with linear regression analysis to calculate Pearson's correlation coefficient. Data are presented as mean ± SE. Significance was set at $p < 0.05$.

RESULTS

Maternal and fetal leptin concentrations

Maternal serum leptin concentration was lower in nOB (48.2 ± 11.4 ng/ml) animals compared to OB (116.4 ± 12.4 ng/ml) ($p < 0.05$). There were no differences in fetal leptin concentrations between two groups [20].

Serum, placental and maternal adipose tissue content of AEA and 2-AG

Placental AEA as well as serum and maternal fat 2-AG concentrations were similar in both groups (Fig. 1). AEA concentration in maternal fat was higher in the obese animals (Fig. 1A). Concentrations of 2-AG in placenta were lower in obese baboons (1254.1 ± 401.3 nmol/kg) vs. non-obese (3124.2 ± 557.3 nmol/kg) (Fig. 1C). There were no correlations between placental/maternal fat and serum ECB concentrations. AEA contents were lower than 2-AG contents in maternal serum and fat (Fig. 1). Maternal serum AEA concentrations correlated negatively with the weight of maternal liver ($r = -0.72$, $p = 0.04$) and also showed slight, but significant correlations with the weights of fetal brain ($r = 0.74$, $p = 0.04$) and fetal pancreas ($r = 0.75$, $p = 0.03$). Maternal fat and placental 2-AG concentrations positively correlated with each other ($r = 0.82$, $p = 0.013$) and with maternal leptin concentrations ($r = -0.72$, $p = 0.04$ and $r = -0.83$, $p = 0.01$ respectively).

Expression of ECB mRNA and proteins in placenta

Expressions of *CB1* and *CB2* receptor mRNA were lower in obese placentas, compared to the non-obese (Fig. 2A). Expressions of *DAGL-A*, *DAGL-B*, *NAPE-PLD*, *MAGL* and *FAAH* did not differ between obese and non-obese animals. Relative expression of CB2 receptor protein was higher in obese compared to non-obese placentas (Fig 2B).

Expression of ECB proteins in the placental villous tree

Localization—Villous trophoblastic cells demonstrated relatively weak expression of *CB1* and *CB2* receptors (Fig. 3A, 3B) and intense expression of *MAGL* and *FAAH* (Fig. 3E and 3F). Villous capillary endothelium expressed *CB1* receptors (Fig. 3A), *NAPE-PLD* (Fig. 3C), *DAGL* (Fig. 3D), and *MAGL* (Fig. 3E).

Histomorphometry—There were no observed differences in the villous and basal plate protein expression of ECB ligands, their receptors, and metabolizing enzymes between obese and non-obese animals (Table 3).

DISCUSSION

Maternal obesity exerts significant morbidities during pregnancy and birth (e.g., stillbirth, pre-eclampsia) and throughout the offspring's lifespan [27]. To our knowledge, this study is the first to report the responses of placental and maternal ECBs to maternal obesity in any model. Subcutaneous fat has been shown to act as an endocrine organ [28] and accounts for almost 90% of total fat deposition in women [29]. ECBs and their receptors are essential regulators of metabolism for multiple cardiovascular, neurologic, reproductive, and immune processes [30, 31]. Dysregulation of ECBs within subcutaneous fat is a hallmark of obesity [32]. Thus, it is reasonable to hypothesize that metabolic cross-talk between placenta and fat could mediate negative consequences of maternal obesity.

Components of 2AG enzymatic pathway

Despite the confirmed role of the ECB system to maintain pregnancy [33], the role of 2-AG in placenta remains unclear [9]. In our current study, 2-AG concentration was 10-fold higher in placenta than maternal serum, suggesting placenta as a source or depot for this key ECB ligand in pregnancy. This observation agrees with data published for rat placenta; however, the placental concentration of 2-AG in the baboons was higher than that reported for rodents [9]. Subcutaneous fat concentration of 2-AG was lower in baboons (vs. humans [32]); but serum values resembled those reported for humans and rodents [18]. In addition to biologic reasons, these differences in absolute concentrations could be attributed, to different analytical methodologies used.

The decrease in placental 2-AG content observed in obese baboons is intriguing. Similar decreases have been described in subcutaneous fat from obese humans [32]. In jejunum, 2-AG accumulation was associated with the dietary consumption of 18:1 oleic acid [34]. Keeping in mind that Syncytiotrophoblast (ST) shares striking structural and functional similarities with the small intestine [35], and also that maternal obesity significantly influences placental oleic acid uptake [36], it is logical to posit that the regulation of placental 2-AG pathways could be coupled to placental fatty acid transport. Recent evidence of association between maternal fat intake and offspring ECBs function in rats supports this idea [37].

Another mechanism for ECB regulation could involve leptin, and maternal concentration of leptin is increased in MO [20]. In brain and subcutaneous fat, leptin regulates energy homeostasis via down-regulation of 2-AG and CB1 receptor expression [38, 39]. In our study, maternal fat and placental 2-AG concentrations correlated directly; 2-AG concentrations in both tissues showed negative correlations with the maternal serum leptin concentration. Similar effect of leptin on 2-AG metabolism has been reported in the pregnant rodent uterus [40], yet the exact mechanism remains unclear. In neural tissues, 2-AG content is regulated by its enzyme-dependent synthesis (via DAGL-A and DAGL-B), as well as degradation (via MAGL) [41]. The enzyme activity has not been measured in our

study, and our data are limited to these proteins per se. MAGL expression was detectable in ST, which is also the site of leptin expression in humans and non-human primates [42]. Notably, proteomic analyses of the ST apical brush border by Vandre et al. [43] revealed presence of another 2-AG hydrolyzing enzyme-ABHD12. ABHD12, plays an important role in brain microglial 2-AG - CB2 interaction and is responsible for 9% of 2AG hydrolysis. Thus ST might be an important site of ECBs hydrolyses. ECB hydrolysis could lead to a described in maternal obesity placental inflammatory response (macrophages and neutrophils accumulation)[20, 44] through the formation of AA and COX-2 [45]. However, in rodents, decreased 2-AG production was protective against inflammatory liver injury [46]. The higher amount of 2-AG—compared to AEA in serum and fat in our study—agrees with data published for other tissues, e.g. brain [47] and decidua [48].

Components of AEA enzymatic pathway

The increase of the AEA in the subcutaneous fat of obese mothers in our study agrees with data from non-pregnant women [6]. In contrast, the serum AEA concentrations did not differ between obese and non-obese animals in our study and were similar to those reported for human pregnancies [18, 49]. Associations between the maternal circulating AEA, weight of maternal liver, and weight of insulin-sensitive fetal tissues (brain and pancreas) are intriguing. The role of AEA as a central player in the regulation of the insulin-sensitivity, including development of Type 2 diabetes has been recently emerged [50].

CB1 and CB2 receptors

The *CB1* receptor is closely involved in pregnancy maintenance, as shown in human miscarriages [18] and in spontaneous preterm labor in CB1 receptor knock-out mice [51]. Down-regulation of placental *CB1* and *CB2* receptors mRNA in MO here agrees with data published for mRNA expression in brown fat of obese Zuckerrats [52] and might provide a mechanism for the epidemiological link between maternal obesity and preterm labor [53]. The protein levels of CB1 receptors as evaluated by WB and IHC did not differ between two groups in our study. The up-regulation in protein expressions of CB2 receptor in WB is in agreement with published data regarding the role of CB2 in inflammation[54]. However, the mechanisms of CB2 regulation of inflammation and energy homeostasis are still remain obscure[54, 55]. The controversies in different published studies as well as our observed discrepancies between mRNA, WB and histomorphometry could result from the complexity of ECB molecular biology[55] and the limitations, associated with each detection. Distinct mRNA transcripts for example, could arise from different isoforms and splicing variants of both receptors [56]. The protein content in WB mirrors the diverse populations of placental cells from maternal and fetal origins, each of which could have distinct regulatory mechanisms and functions. Mitochondria express both *CB1* and *CB2* receptors [57]. The control of mitochondrial function by ECBs may influence placental metabolic rate and therefore fetal development. For example, changes in placental metabolism related to higher altitude have been described as a cause of fetal growth retardation [58]. The localization of *CB1* and *CB2* receptors in trophoblast makes these receptors ideal candidates for regulation of placental metabolism [59].

In summary, the present study demonstrated for the first time the differential regulation of the ECBs in maternal fat and placental tissue related to maternal obesity. Differential regulation and function exists for the AEA and 2-AG pathways in both tissues, with decreased 2-AG concentration in placenta and increased AEA content in the subcutaneous fat from obese mothers. Our data suggest these pathways might exert different roles during fetal development.

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Figure 1.

Figure 2.

Figure 3.

Table 1

Weight of maternal and fetal organs of obese and non-obese baboons (*Papio*spp) (Data presented as mean $\pm$ SE).

	Non-Obese <i>n</i> =4	Obese <i>n</i> =4
Weight of maternal organs		
Heart (g)	61.7 $\pm$ 2.09	57.3 $\pm$ 7.45
Lungs (g)	98.48 $\pm$ 7.55	88.6 $\pm$ 3.43
Thymus (g)	3.26 $\pm$ 0.39	3.06 $\pm$ 0.89
Thyroids (Combined) (g)	1.49 $\pm$ 0.33	1.45 $\pm$ 0.39
Liver (g)	292.00 $\pm$ 19.61	319.55 $\pm$ 23.14
Spleen (g)	19.38 $\pm$ 1.76	21.17 $\pm$ 3.00
Pancreas (g)	17.42 $\pm$ 3.00	16.7 $\pm$ 1.87
Kidney (combined) (g) *	72.00 $\pm$ 2.00	65.7 $\pm$ 2.00 ^A
Brain (g)	134.69 $\pm$ 3.09	132.34 $\pm$ 11.00
Weight of fetal organs *		
Heart (g)	4.4 $\pm$ 0.6	4.5 $\pm$ 0.5
Lungs (g)	23.01 $\pm$ 2.94	22.28 $\pm$ 3.63
Thymus (g)	3.42 $\pm$ 0.34	3.78 $\pm$ 0.44
Thyroids (Combined) (g)	0.29 $\pm$ 0.09	0.31 $\pm$ 0.04
Liver (g)	24.93 $\pm$ 1.57	25.36 $\pm$ 4.85
Spleen (g)	1.61 $\pm$ 0.10	1.54 $\pm$ 0.15
Pancreas (g)	0.66 $\pm$ 0.10	0.56 $\pm$ 0.22
Kidney (Combined)	2.58 $\pm$ 0.17	2.02 $\pm$ 0.23
Brain (g)	80.22 $\pm$ 1.54	76.1 $\pm$ 6.88

^A $p < 0.05$ compared to non-obese group.

* Data obtained from [20].

Table 2

Primers, probes and conditions (A), antibodies and blocking peptides (B) used.

A. Q-RT-PCR conditions			
Transcripts	Left(l) and Right(R)Primers	Probes*	Reactionconditions**
<i>CB1</i>	L 5'-AAAATTCTGTAATGTTACCTGGTC-3' R 5'-AAAATTCTGTAATGTTACCTGGTC-3'	n/a	95°C-20sec 58°C- 30sec 72°C- 20sec
<i>CB2</i>	L 5'-GGAGAGGACAGAAAACAACG-3' R 5'-GAGCTTGCTAGAAAGGCTTTGG-3'	n/a	95°C-20sec 58°C- 30sec 72°C- 20sec
<i>NAPE PLD</i>	L 5'-AGATGGCTGTAATGGGAA-3' R 5'-TTCTCCTCCCACAGTC-3'	n/a	95°C-15sec 58°C- 30sec 72°C- 10sec
<i>MAGL</i>	L 5'-AGCTGACTGTGCCCTTCCT-3' R 5'-GGTAGGCACCTTCATAAATCTTG-3'	#1,cat.no.04684974001	95°C-15sec 55°C- 30sec 72°C- 10sec
<i>DAGL- A</i>	L 5'-AAGCACCAAGCCCAAATG—3' R 5'-CTCAGGCAGCTCCGACTT—3'	#49,cat.no.04688104001	95°C-15sec 55°C- 30sec 72°C- 10sec
<i>DAGL B</i>	L 5'-GCCTTGCTCAGCAGTTACTTG-3' R 5'-CCAGGGTTACAAATCGTTCC-3'	n/a	95°C-15sec 55°C- 30sec 72°C- 10sec
<i>FAAH</i>	L 5'-CTCTGCTGCCAAGGCTGT-3' R 5'- TGCAGTTCCAGAGTTTCC-3'	#73,cat.no.04688961001	95°C-15sec 55°C- 30sec 72°C- 10sec
<i>-ACTIN</i>	L 5'-CCAACCGCGAGAAGATGA-3' R 5'-CCAGAGGCGTACAGGGATAG-3'	#64,cat.no.04688635001	95°C-15sec 55°C- 30sec 72°C- 10sec

B. Antibodies and blockingpeptides (IH)				
	Catalognumber	Company	Host	Dilution
CB1	SP5262P	AcrisAntibodiesGmbH, Herford, Germany	Rabbit	1:1000(IH, WB)
CB2	H00001269-MO1	Abnova, Taipei City, Taiwan	Mouse	1:500(IH),1:2000 (WB)
NAPE PLD	10305 Blockingpeptide: 10303, dilution 1:5	CaymanChemical, Ann Arbor, MI, USA	Rabbit	1:50 1:50
MAGL	100035 Blockingpeptide 300014, dilution 1:1	CaymanChemical, Ann Arbor, MI, USA	Rabbit	1:50
DAGL	sc-133307, Blockingpeptide sc-133307, dilution 1:5	Santa Cruz Biotechnology, Inc., Dallas, TX, USA	Rabbit	1:50
FAAH1	ab54615	Abcam, Cambridge, MA USA	Mouse	1:500
<i>-ACTIN</i>	A3854	Sigma, St. Louis, MS, USA	Mouse	1:20000 (WB)

Abbreviations: IH- Immunohistochemistry; WB-Western Blot

Table 3

Endocannabinoid system staining intensities (calculated as histoscore) in placentas from obese and non-obese baboons (*Papio*) (Data presented as Mean $\pm$ SE)

Antigen		Obese (n=4)	Non-Obese (n=4)	p values
CB1	Villi	53.75 $\pm$ 19.2	47.01 $\pm$ 25.2	0.84
	Basal plate	63.78 $\pm$ 24.5	47.2 $\pm$ 25.2	0.654
CB2	Villi	95.50 $\pm$ 21.7	96.18 $\pm$ 9.5	0.98
	Basal plate	76.87 $\pm$ 5.2	53.5 $\pm$ 11.6	0.17
FAAH	Villi	7.48 $\pm$ 1.9	3.64 $\pm$ 0.8	0.12
	Basal plate	0.3 $\pm$ 0.1	1.19 $\pm$ 0.5	0.14
MAGL	Villi	232.6 $\pm$ 9.0	250.30 $\pm$ 5.3	0.14
	Basal plate	251.53 $\pm$ 9.0	253.20 $\pm$ 20.0	0.94
DAGL	Villi	150.97 $\pm$ 14.7	157.15 $\pm$ 23.2	0.83
	Basal plate	53.10 $\pm$ 13.11	93.30 $\pm$ 17.3	0.12
NAPE-PLD	Villi	163.19 $\pm$ 8.7	181.16 $\pm$ 18.6	0.42
	Basal plate	193.75 $\pm$ 30	168.74 $\pm$ 19.64	0.51