From DEPARTMENT OF LABORATORY MEDICINE, DIVISION OF PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

CHORANGIOMAS: HISTOPATHOLOGICAL, CLINICAL AND GENETIC STUDIES

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Stockholm 2017

Cover illustration by Meeli Sirotkina.
Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7676-828-0

Karolinska Institutet

Institutionen för Laboratoriemedicin, Avdelningen för Patologi

Chorangiomas: histopathological, clinical and genetic studies

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Hörsal 4Z, plan 4, Alfred Nobels allé 8, campus Flemingsberg

Fredagen den 10 november 2017, kl 09.00

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Chorangiomas: histopathological, clinical and genetic studies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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“Do not go where the path may lead, go instead where there is no path and leave a trail.”

-R. W. Emerson

In memory of my father, Vladimir Sirotkin

ABSTRACT

Chorangioma (CA), although, is the most common non-trophoblastic, vascular, tumor-like-lesion of the placenta with incidence approximately 0.5-1% of all examined placentas, the specific etiology and genetic background of these lesions is still poorly understood. However, an increased incidence of CAs has been reported in pregnancies occurring at high altitudes and in relation to the in utero hypoxic status (preeclampsia, multiple gestation). Thus, CAs are suggested to be hypoxia-induced reactive vascular hyperplasias rather than true tumors, although this theory has not been supported by systemic genetic studies, so far. In contrast, the occasional reports of recurrence of CA suggest that genetic factors may play also role in the pathogenesis of these lesions. Further, infantile hemangioma (IH) shares various histochemical and genetic characteristics with placental endothelial cells; notably, a predictable life cycle of initial proliferation followed by apoptotic involution similarly to that of the placenta. These findings suggest the possibility that the placenta could be the origin site of IH.

Our first and second studies characterized 170 CA cases morphologically and clinically providing evidence that CAs are associated with an increased rate of hypoxia related placental morphological changes and more adverse clinical outcome in singleton pregnancies compared with multiple pregnancies.

Our cohort of CAs demonstrated a high incidence of preeclampsia, which could be an invaluable information for clinical placental diagnostics and might lead to a possible recognition of CAs as potential morphologic indicator for placental hypoxia.

The third study of genetic background of CA analyzed eight large CAs using the array comparative genomic hybridization method and revealed no pathogenic copy number variants in the CA samples compared with either standard control DNA or unaffected placenta DNA from the same individual. This lack of association in our pilot study could support a non-tumorous, non-genetic origin of the CAs; however, additional genetic studies of larger sample sets are required to fully exclude a possible genetic contribution.

In our fourth study, we investigated the co-existence of CA and IH using a questionnaire answered by the parents and failed to demonstrate any correlations between CAs and IH. Furthermore, the occurrence of multiple pregnancies or preeclampsia was not associated with an increased incidence of IH. The latter could be explained by the fact that pathogenesis of IH is more complex and several risk factors contributing to its etiology.

LIST OF SCIENTIFIC PAPERS

I. **Meeli Sirotkina,** Konstantinos Douroudis, Magnus Westgren, Nikos Papadogiannakis.

Association of chorangiomas to hypoxia-related placental changes in singleton and multiple pregnancy placentas. Placenta. 2016;39;154-9.

II. **Meeli Sirotkina,** Konstantinos Douroudis, Nikos Papadogiannakis, Magnus Westgren.

Clinical Outcome in Singleton and Multiple Pregnancies with Placental Chorangioma. PLoS One. 2016;11(11):e0166562.

III. **Meeli Sirotkina,** Magnus Westgren, Nikos Papadogiannakis.

Genetic analysis of copy number variation in large chorangiomas. Manuscript.

IV. **Meeli Sirotkina,** Konstantinos Douroudis, Carl-Fredrik Wahlgren, Magnus Westgren, Nikos Papadogiannakis.

Exploring the association between chorangioma and infantile hemangioma in singleton and multiple pregnancies: a case control study in a Swedish tertiary centre. BMJ Open 2017;7:e015539.

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LIST OF ABBREVIATIONS

a-CGH	array comparative genomic hybridization
BFC	blood flow classes
CA	chorangioma
CI	confidence intervals
CNV	copy number variant
dbSNP	the single nucleotide polymorphism database
Flt-1	fms related tyrosine kinase 1
GLUT1	glucose transporter protein isoform 1
hCG	human chorionic gonadotropin
HELLP	hemolytic anemia, elevated liver enzymes and low platelet count
HIF-1	hypoxiainducible factor 1
hPL	human placental lactogen
IGF2	insulin-like growth factor 2
IH	infantile hemangioma
IUGR	intrauterine fetal growth restriction
LGA	large for gestational age
MRI	magnetic resonance imaging
NICH	non-involuting congenital hemangiomas
OR	odds ratio
PI	pulsatility index
PlGF	placental growth factor
RICH	rapidly involuting congenital hemangioma
SD	standard deviation
SGA	small for gestational age
sFlt-1	soluble fms-like tyrosine kinase-1
SNOMED	Systematized Nomenclature of Medicine
SNV	single nucleotide variant
TGF-β1	transforming growth factor-β1
TEM8	tumor endothelial marker-8
VEGF	vascular endothelial growth factor
VEGFR1	vascular endothelial growth factor receptor 1
VEGFR2	vascular endothelial growth factor receptor 2
VLA4	very late antigen 4
WGS	whole genome sequencing

1 BACKGROUND

1.1 CHORANGIOMA (CA)

1.1.1 Introduction

Chorangioma (CA) is the most common non-trophoblastic, vascular, tumor-like-lesion of the placenta, first described by John Clarke in 1789 (1). In the early stage of pregnancy, development of the normal placenta begins with vasculogenesis (the *de novo* formation of blood vessels from progenitor cells) and formation of precursor villi, where endothelial progenitor cells differentiate into angioblastic cords (2,3,4). These precursor villi form later the stem villi. The early placental development is influenced by several factors, including local low oxygen tensions, high level of growth factor receptors and their ligands (in particular, vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) (2,5,6). Low oxygen environment is result of extravillous trophoblast invasion into the decidua, occluding spiral arteries and restricting blood flow into the intervillous space (7). Further, the capillaries are formed by angiogenesis, where new vessels arise through branching of preexisting vessels and give rise to the immature intermediate villi (2,3). Noteworthy, hypoxia is the main pathophysiologic stimulus for angio-and vasculogenesis (8). Later, the process transforms to a non-branching linear growth because of high oxygen tension, as spiral arteries become patent and blood flow increases, and low VEGF levels, to form mature intermediate and finally terminal villi (2,5,7). CA is a lesion of capillary dysvasculogenesis and they are usually found at the placental margin or immediately below the chorionic plate, which are the most poorly perfused areas of the placenta (2,3). Therefore, several possibilities for pathogenesis are suggested, including: inception early in gestation, origin from primary stem villi, or induced by hypoxia (3). Origin early in gestation seems unlikely in view of the lack of reported cases in early pregnancies (3). Ultrastructural studies support the origin from primary stem villi (3). An increased incidence of CAs has been reported in pregnancies occurring at high altitudes and in relation to maternal smoking (2,3). Consequently, it has been suggested that CAs may be hypoxia-induced vascular proliferations influenced by multiple growth factors and subsequently associated to the pathological conditions during pregnancy rather than to abnormal embryogenesis (1). Thus, CAs are hypothesized to be reactive vascular hyperplasias or hamartomas rather than true tumors, although this hypothesis has not been supported by systemic genetic studies so far (3,9).

1.1.2 Incidence

The incidence of CA is reported approximately 0.5 and 1% of all examined placentas (1,10). It varies in different reports about different study cohorts. Guschmann et al. performed a retrospective study on 22439 supposedly unselected multiple and singleton placentas and found 136 cases of CA, with an incidence of 0.61% (11,12). Wou et al. examined retrospectively 14725 singleton placentas and found 23 cases with CA; the incidence was

0.16% (11,13)**.** Ogino et al reported 36 cases of CA in a cohort of 7062 examined placentas, incidence of CA was 0.51% (3).

Most CAs are incidental findings measuring less than 5 mm (14). Lesions up to 40 mm in diameter are usually asymptomatic and the clinical significance of microscopic CAs remains unknown (14). Large CAs measuring more than 4–5 cm, with an incidence of 1:3500 to 1:9000 (0.29%–0.11%) births (1,11,13,15,16,17,18,19,20). CAs are supplied by the fetal circulation and are often associated with maternal and fetal complications including polyhydramnios, antepartum hemorrhage with premature placental detachment, preterm labor, fetal anemia, thrombocytopenia, non-immune fetal hydrops, intrauterine fetal growth restriction (IUGR) and increased perinatal mortality (11,14,21,22,23,24,25,26). The overall mortality rate associated with large CAs is about 20-40% (6,7,87,89,91). Although the underlying pathophysiology for these complications has not been clearly elucidated, several mechanisms explaining the pathogenesis of the complications are proposed. For example, a chronic arteriovenous shunting in the tumor can affect the fetal circulation by increasing the venous return to the heart and causing the development of fetal congestive heart failure, which could be suspected by the presence of hydrops (11,23,24,25,30,31). The sequestration of red blood cells and platelets within the tumor and thrombocytic microangiopathy are, in turn, the main hypotheses, that could explain the pathogenesis of anemia and thrombocytopenia (11,20,23,24,25,31). Further, polyhydramnios has been associated to increased production of urine due to the hyperdynamic fetal circulation linked either to shunting of the blood in CA or fetal anemia (19,20). In addition, the transudation of fluid from CA surface may contribute to the accumulation of amniotic fluid (19,20).

The presence of CA correlates with increased maternal age and has been associated with HELLP (hemolytic anemia, elevated liver enzymes and low platelet count) syndrome, preeclampsia, multiple gestation and preterm birth (3,11,23). CAs can be usually observed from the second trimester of pregnancy (3,11).

It is reported that CA occur more often in the female fetuses (6,12,13,32). Guschmann et al. analyzed 136 cases of CA and the female fetus was related in 72.2% of pregnancies (11,12). Comparably, Wou et al. reported that in 16 out of 23 (69.6%) CA cases female neonates were involved (11,13)**.**

An increased incidence of CA has been described also in pregnancies at high altitude (above 3600 m) where the most notable factor of all specific environmental conditions is the prevailing hypobaric hypoxia (2,3,11). Hypoxia is believed to be a major stimulus for placental angiogenesis (2,33,34). The placenta at high altitude is capable to increase its oxygen-transporting capacity between mother and fetus through the reduction in the thickness of the vasculosyncytial villous membranes as a result of adaptation to the hypobaric hypoxia (34,35). Of interest, over-expression of angiogenic cytokines, such as VEGF, which is known to be up-regulated by hypoxia, may lead to excessive proliferation of endothelial cells and formation of CA (11,35).

The recurrence of CA in subsequent pregnancies has been reported in occasional studies, suggesting that genetic predisposition may also play a role in the pathogenesis of CA (2,11,36).

1.1.3 Gross morphology

CAs are usually single, but multiple lesions can be found after thorough morphological examination of the placenta (1,37). They may be small, a few millimeters in diameter, or may form large masses, even up to 1500 g (1,2). Large CAs are most commonly seen as nodular lesions bulging on the fetal surface of the placenta (1,2,23) (Figure 1.). Occasionally they may be attached to the placenta only by a vascular pedicle (1,2). Most CAs are small, within the placental substance and not visible externally, hence to be detected the placenta must be systematically sliced (1). The small intraplacental lesions are usually round and well demarcated from the surrounding normal placental parenchyma (1). The cut surface of CAs may vary from red to white and is usually smoother and firmer than surrounding placental tissue (1). They may resemble fibromas, when a large stromal component is present (2). The small CAs can be distinguished from intervillous thrombi by the absence of lamination (1).

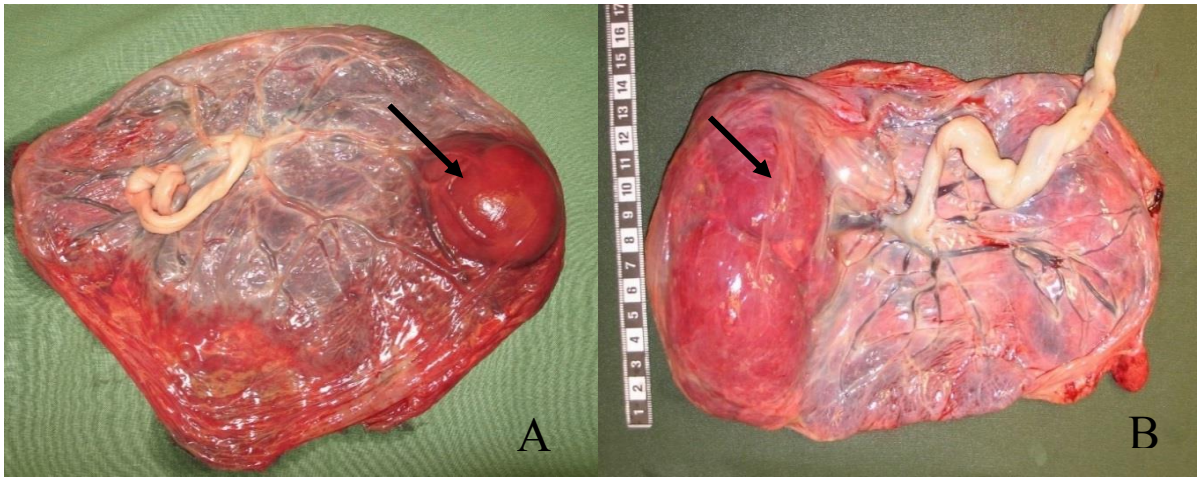

Figure 1. Gross morphology of large CAs (arrows, A, B).

1.1.4 Microscopic features

Most CAs have histological appearance analogous to hemangiomas at other body sites (hemangioma of the placenta) (1,2,3). CA is a well-demarcated nodular mass composed of numerous capillary vascular channels supported by a perivascular stroma containing fibroblasts, macrophages and scanty fibrous tissue (1,2,3,38) (Figure 2.). They are surrounded by a layer of trophoblast (2,3). The stromal component may be predominant with very few vessels and may show degenerative changes such as myxoid changes, hyalinization, necrosis, calcification, which may complicate and confuse the histological appearance (1). Mitotic figures may be seen in some cases of CA and they may be associated with some degree of

atypia in endothelial or stromal cells; there is, however, no evidence that CAs behave as malignant tumors (1,30). Immunohistochemically CA cells show positive expression for CD31, CD34, factor VIII, GLUT 1 (glucose transporter protein isoform 1) and cytokeratin 18 suggesting origin from blood vessels of the chorionic plate and stem villi (23,39)

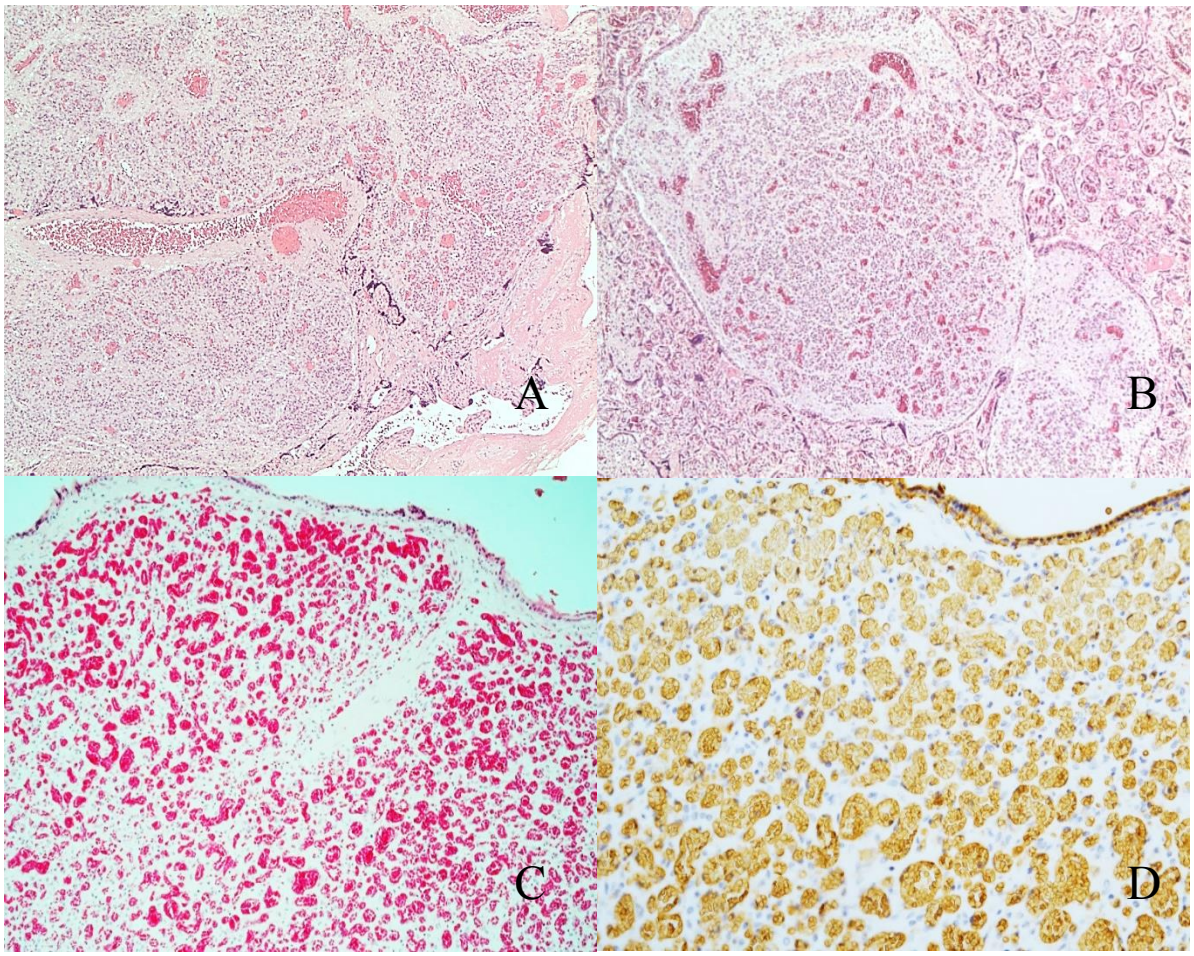

Figure 2. Histological features of large (A) and microscopic CA (B), hem.-eos, 50x; nodular structure and proliferation of capillary blood vessels in CA (C), hem.-eos. 100x, GLUT 1 positive staining of endothelial cells and erythrocytes in CA (D), 200x.

1.1.5 Molecular background

Very little is known about the genetic background of CAs, however, the occasional reports of recurrence of CA suggest that genetic factors may play role in the pathogenesis of these lesions (2,11,36). Very few cytogenetic data have been reported regarding CA in the literature. Miliaras et al reported deletions at 2q13 and 7p21.1 in CA and hepatic cavernous hemangioma tissue presented in the same patient (40,41). Wuster et al found a chromosomal translocation at 2q-;15q+ in dizygous twin girl with mental deficiency and marked placental chorangiomatosis (42). Kim et al, however, reported a normal karyotype in another case of CA (43).

1.1.6 Diagnosis

Small CAs are usually found only after careful morphological examination of the placenta (15,26). Large CAs, however, are in most cases found prenatally during routine ultrasound examination and diagnosis is confirmed by histopathologic examination (15,22,23). Gray-scale ultrasound shows hypo-or hyperechogenic well-circumscribed mass, which is clearly different from surrounding placental tissue (26,44,45). The ultrasonographic echo-texture of CA is usually changing during pregnancy due to the degenerative changes (calcification, necrosis) in the tumor (23,44). By color Doppler imaging vascular nature with blood flow within the lesion can be confirmed and the relation to fetal circulation elucidated (29,31,44,46). Feeding vessels of the lesion usually show same pulsatile flow as that of umbilical artery arteriovenous shunting in the tumor, however, can cause low resistance flow (30). Routine ultrasound examinations, including gray-scale and color Doppler ultrasonagraphy, plays an important role in the prenatal diagnosis and monitoring of large CAs (15,44). Due to the potential risk for complications caused by the CA, early diagnosis, close prenatal surveillance, and intrauterine treatment can be implemented to prevent fetal morbidity and mortality (15,44,47).

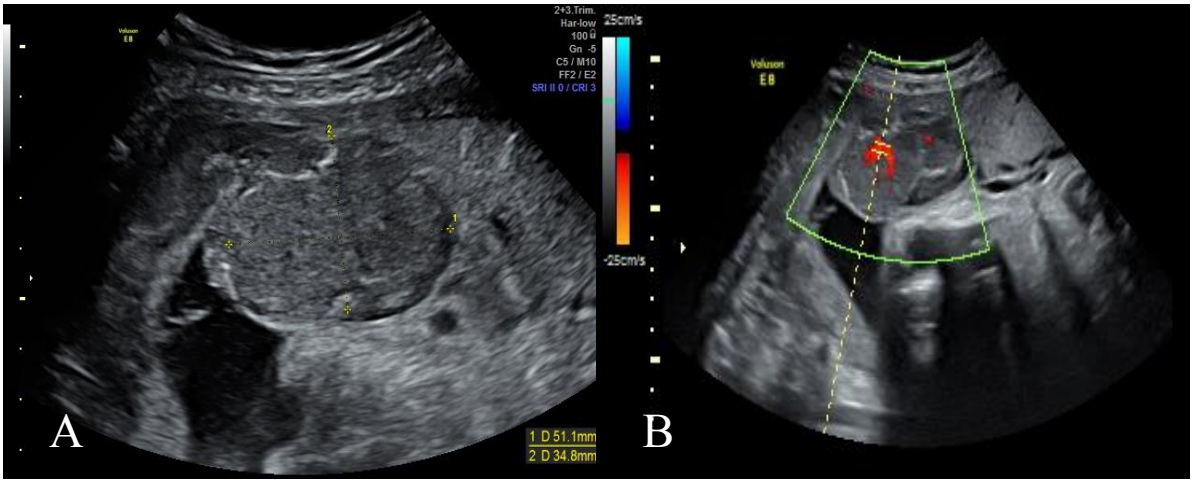

Figure 3. Gray-scale ultrasound image of well-demarcated large CA at gestational age 34+5 weeks (A); color Doppler image, same case (B).

1.1.7 Treatment

Approximately 50% of large CAs develop complications requiring clinical intervention (48). Arterio-venous shunting in the large CA can create significant hemodynamic disturbances in fetal circulation and result in fatal complications (48). Thus, the treatment of CAs should be targeted to diminish or arrest the blood flow in the CA (29,48). Eleven cases of fetoscopy-guided laser photocoagulation of surface feeding vessels and 7 cases of antenatal embolization of the tumor feeding vessels using alcohol, microcoil or cyanoacrylate have been reported regarding attempts to treat large CAs (10,16,19,28,48,49,50). Based on reported treated cases, successful result of the clinical intervention is achieved, when CA is

located further away from the umbilical cords insertion, so that the main circulation of the tumor is not directly dependent on the umbilical cord. (10)

Also, endoscopy-guided surgical ligation of vessels with bipolar electrocautery and ultrasound-guided percutaneous interstitial laser ablation have been described as therapeutic methods for large CAs (28,31,48).

Complications secondary to large CAs as polyhydramnios and fetal anemia have been treated with therapeutic amnioreduction, which is the least invasive prenatal intervention option, and intrauterine blood transfusion (18,28,29,30,45,48).

Padys et al suggested a non-selective beta-blocker, propranolol, which has been used to treat fetal tachycardia and inhibit the growth of infantile hemangioma, as an alternative to invasive procedures for the treatment of large CAs (17,51).

Management of large CAs remains, however, a therapeutic challenge (10).

1.2 INFANTILE HEMANGIOMA (IH)

1.2.1 Introduction

Infantile hemangioma (IH) is the most common neoplasm of childhood (52,53,54). Its exact etiology and pathogenesis remains unclear despite thorough studies and many proposed mechanisms (53,55,56). Nevertheless, angiogenesis and in recent studies even vasculogenesis are suggested to play an important role in pathogenesis of these lesions (57,58). IHs are benign vascular tumors characterized by a predictable clinical course in form of a unique ability to involute after rapid proliferation (52,53,59). The initial proliferative phase begins in the first 2 weeks of life and lasts 6 to 10 months, when tumor grows rapidly due to an excessive proliferation of endothelial cells (52). This is followed by gradual spontaneous involution, when proliferation is reduced and apoptosis increases, thus, the growth of the lesions slows down and finally stops (52). The involuting phase, with histological fibrosis and fat deposition, begins between 6 and 12 months of age and most of tumor regression occurs before age of 4 years (53,59). An intermediate phase, between proliferation and involution, more likely represents a period of temporary balance between cellular proliferation and involution/apoptosis (53). Approximately 50% to 70% of IHs undergo spontaneous regression but resolve incompletely, leaving permanent residual skin changes including fibro-fatty residua, telangiectasia or dyspigmentation (53,59). Only about 10-20% of IH become clinically significant and require treatment (52,57,59).

There are also rare congenital variants of the common infantile hemangioma, with similar features, but biologically and behaviorally distinct forms (52,53,54). Congenital hemangiomas are fully formed at birth and do not express GLUT1 (52,53,54,60). The rapidly involuting congenital hemangiomas (RICH) regress rapidly within the first year of life (7-14

months) (52). In contrast, non-involuting congenital hemangiomas (NICH) remain stable after birth without growth or regression (52,53).

1.2.2 Incidence

The estimated incidence of IH is approximately 3-10% of infants (61). About 60-80% of IHs occur on the head and neck (52,62). Female infants are more likely to be affected; female-to-male ratios varying from 1.4:1 to 3:1 and a significantly higher incidence of IH has been reported in Caucasian infants (53,54,59,63). It has been suggested, that low birth weight, which is usually related also to prematurity, is the major contributor to an increased risk of IH, affecting 22-30% of infants weighing less than 1 kg and with every 500-g reduction in birth weight the risk of developing IH increases 25% (53,59,63,64). The mechanism, by which fair skin and female neonates show an increased risk of developing IH might be related to the higher levels of renin compared with dark skin and male neonates (65). An increased level of renin leads to a higher level of angiotensin II within the IH, which in turn promotes the endothelial proliferation (65). Increased renin level is found also in Caucasians compared with Blacks, premature compared with full-term infants, and children compared with adults (65). It could even explain the spontaneous involution of IH: the renin levels decrease as children grow older (65).

Of interest, Kim et al reported correlation between unplanned pregnancies with maternal smoking and/or alcohol consumption and increased incidence and severity of IH (66).

In addition, higher prevalence of IH has been described in relation to several prenatal factors including increased maternal age, preeclampsia, multiple gestation, placenta praevia and breech presentation. Further, placental abnormalities such as retroplacental hematoma, infarction, dilated vascular communications and in association with previous trans-cervical chorionic villous sampling or amniocentesis, have also been associated with IH development (53,59,65). It has been hypothesized that the common link in these associations might be placental hypoxia. (53).

1.2.3 Gross morphology

Proliferative phase may start as localized macular teleangiectatic erythema, which becomes more elevated and rubbery in consistency due to endothelial cell continuous proliferation (53). During rapid proliferation, also ulceration of the lesion may arise causing pain and scarring (53). In involutive phase IH flattens and shrinks starting from the center outwards leaving in most cases behind permanent residual skin changes (53).

IHs can be classified by location in soft tissue as superficial and deep (53). Superficial IHs are red from the surface with little or no subcutaneous component (53). They appear earlier

and start to involute sooner than deep IHs which reside deep in the subcutaneous tissue and show bluish surface (53).

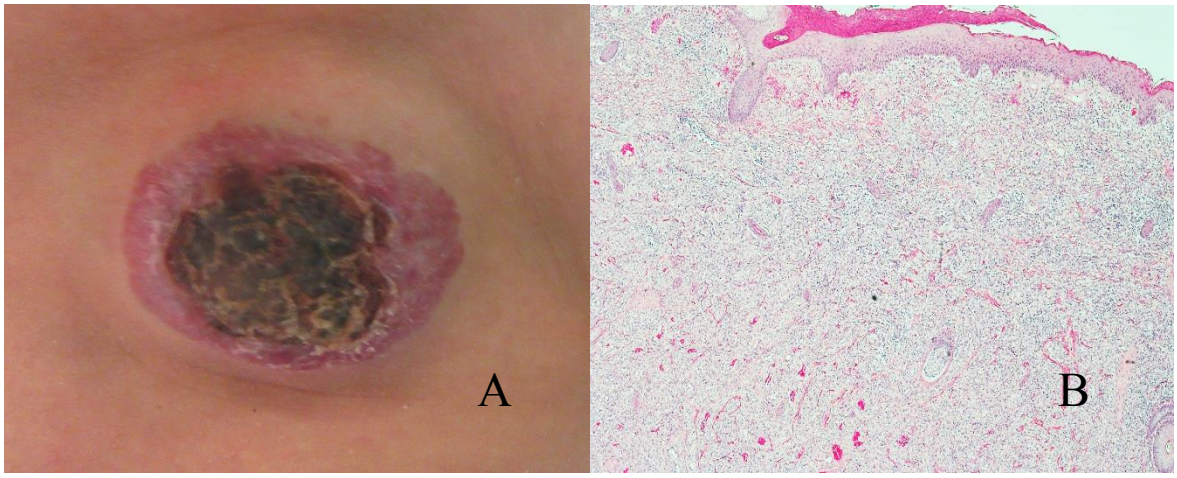

Figure 4. Gross morphology of centrally ulcerating infantile hemangioma (A); histology of proliferating capillary vessels in infantile hemangioma hem.-eos. 40x.

1.2.4 Microscopic features

Proliferative and early involutive IHs are usually well-defined, red nodules or plaques (53). Regressive lesions are less defined, lighter in color and softer, fibro-fatty in consistency (53). During the intermediate phase IHs may show mixture of morphological features of involutive and proliferative phases, as the dividing line between proliferation and involution is not always sharp (53). Histologically the proliferative phase is characterized by well-circumscribed but unencapsulated, lobulated lesion of closely packed capillaries lined by endothelial cells and pericytes, which are separated by delicate fibrous stroma (53). During this phase, endothelial cells and pericytes proliferate actively; hence, normally configured mitotic figures are relatively numerous (53). In involuting phase proliferation of the cells and the number of mitotic figures decreases while the amount of apoptotic bodies and masts cells increases (53). Further, the lesional capillaries start to vanish; the basement membranes hyalinize, may contain apoptotic debris until eventually only loose fibrous or fibro-adipose stroma remains, containing isolated "ghost" capillaries with residual, thickened, hyalinized rinds of basement membrane material studded with apoptotic debris, but without intact endothelial linings (53). Thrombosis or prominent inflammation is not related to regression process (53).

Complementary to clinical history and histological examination the most useful immunohistochemical marker in the diagnosis of IH is GLUT1, which shows strong expression in IH endothelial cells at all stages of their development and therefore may be used as diagnostic tool distinguishing diagnostically challenging variants of IH from other vascular proliferations or neoplasms (53,67).

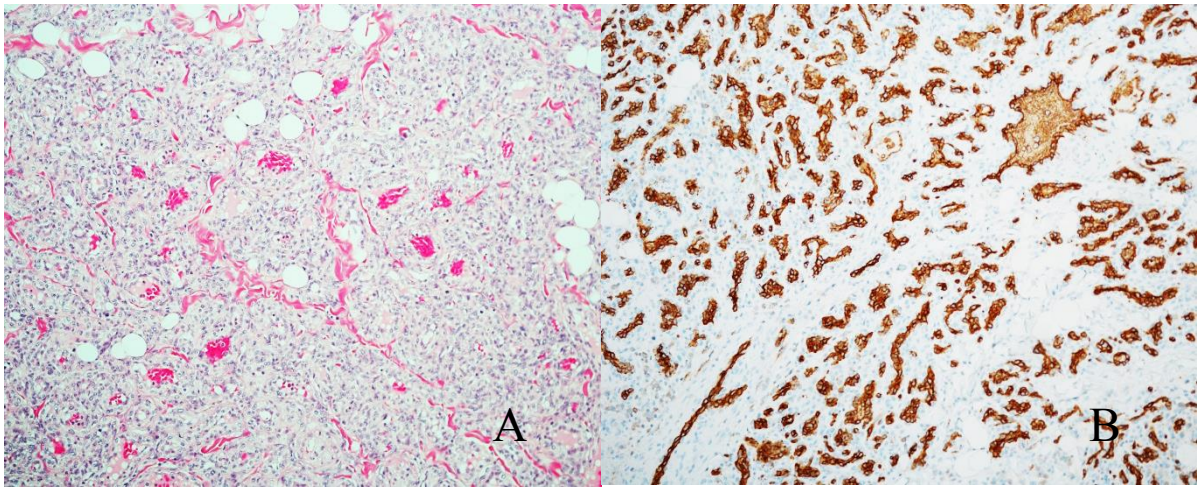

Figure 5. Histology of proliferative capillary blood vessels in the IH (A), hem.-eos. 100x; GLUT 1 positive staining of endothelial cells in IH, 100x.

1.2.5 Molecular background

IHs occur usually sporadically and their molecular basis is unknown (62). However, the occasional reported familial cases of IH and epidemiological studies suggest possible genetic background influencing the pathogenesis of the lesions (62). Five families with autosomal-dominant inheritance of IH have been reported, which allowed the identification of a possible locus on *5q31-33* containing three candidate genes (62). Recent studies demonstrate that some IH patients carry heterozygous missense mutations in the gene *VEGFR2* (Vascular endothelial growth factor receptor 2) or *TEM8 (*tumor endothelial marker-8) (62,68).

1.2.6 Diagnosis

IH is generally diagnosed by history and clinical appearance, imaging of IH is usually not necessary (53,59). However, in cases of uncertain diagnosis, ultrasonography is a reasonable initial method revealing a well-circumscribed high-flow mass with possible vascular shunting (53,59). Echogenicity of the lesion increases during the involution due to the fat tissue replacement (53). Gray-scale ultrasound and color Doppler imaging examination are also first line choices for monitoring the response of IH to pharmacological therapy (53). However, MRI (magnetic resonance imaging) is more useful modality for evaluating the extent of the lesion and the surrounding anatomy (53,59).

1.2.7 Treatment

IHs have uniquely dynamic lifecycle and many lesions involute completely without significant sequelae (69). Approximately 10-20% of patients require clinical intervention (52,70,57,59). Indications for treatment include: 1. IHs causing ulceration, pain and bleeding

2. location in functionally sensitive areas (eyes, airways) and 3. large IHs with significant disfigurement (59,69). The treatment is currently pharmacological (beta- adrenergic blockers, corticosteroids) or surgical (laser therapy, excision in appropriate cases) (69). The beta-blocker propranolol is the first line therapeutic choice in patients without contraindications, including hypotension, heart failure, sinus bradycardia, bronchial asthma and hypersensitivity (53,59,69,71,72,73). The specific mechanism of propranolol`s effect is unknown but more likely is a combination of vasoconstriction, inhibition of angiogenesis and simulation of apoptosis (4,53,59,71,72). Propranolol has shown better effect and fewer side effects than corticosteroids (74). Corticosteroid therapy can be systemic, topical or intralesional and it affects the growth of IH by modifying the pro-angiogenic genes (4,53,69,).

Laser therapy does not inhibit the proliferation of IHs and is mainly used for lesions with non-complete involution and residual teleangiectasia for fading effect (59,69).

Surgical treatment has limited indications: 1. failed or contraindicated pharmacological treatment, 2. anatomical location favorable for resection, 3. high probability that resection will be necessary in the future and the scar will be same regardless of timing (53). Surgical intervention of IH is suggested after age 4 years when involution of the tumor has completed (53).

1.3 PATHOGENETIC THEORIES OF CA AND IH

The specific etiology and pathogenesis of IHs and CAs are still poorly understood. However, indications of the *in utero* hypoxic status (preeclampsia, multiple gestation, placental abnormalities) are reported in cases of CA as well as in cases of IH (3,14,53,75). Also, *in utero* hypoxia is often related to low birth weight, which is known as a major risk factor for IH (53). Hypoxia in general is recognized as an important stimulus of activating angio-and vasculogenesis through an increased expression of various angiogenetic factors, such as, vascular endothelial growth factor (VEGF), placental growth factor (PlGF), Flt-1 (fms related tyrosine kinase 1, a high-affinity transmembrane receptor for both PlGF and VEGF), and sFlt-1 (a placental-derived, truncated, soluble form of Ftl-1) (14,75,76). Therefore, placental hypoxic status or *in utero* hypoxia may act as a trigger in activation of the leader endothelial cells, to initiate a cascade of reactions leading to a proliferation of the endothelial cells and development of IH and/or CA (75). Hence, IH most probably develops as a reaction to the hypoxic environment and endothelial proliferation is a homeostatic attempt to normalize tissue hypoxia (57,78). Furthermore, unlike other vascular proliferations or malformations, endothelial cells in IH show cellular expression of GLUT-1, which also is an important marker of hypoxia and is upregulated through hypoxia inducible factor-1α (HIF-1α) (76,77,78). Consequently, GLUT-1 has been reported to be upregulated within hypoxic zones of tumors and also expressed in the placental endothelial tissue (55,76,77,78).

The unique self-limited growth of IH including rapid proliferation of endothelial cells, followed by apoptosis of the cells and tumor involution may be considered as reflection of the life cycle of placenta, in which endothelial cells are also destined to proliferate for only 9 months (79). Thus, placental origin of the IH as uncontrolled proliferation of embolized cells expressing a phenotype similar to that of placental endothelial cells, has been discussed (14,79). Moreover, placental origin would explain the unique resemblance in immunohistochemical phenotype between vasculature of IH and the placenta, as they share several antigens: GLUT1, merosin, Lewis Y antigen, and Fcγ-RIIb, type III iodothyronine deiodinase, indoleamine 2,3- deoxygenase, and insulin-like growth factor 2 (IGF2) (54,79). Furthermore, similarities have been described also in transciptomes of human placenta and IH (55). Genes preferentially expressed in both placenta and IHs were identified, including 17-β hydroxysteroid dehydrogenase type 2 and tissue factor pathway inhibitor 2 (54,77,79). Further, expression of β-human chorionic gonadotropin (hCG) and human placental lactogen (hPL) and the absence of the trophoblast markers by endothelium of proliferating but not involuting IH, suggest that IH may origin from a placental chorionic villous mesenchymal core cells rather than from trophoblast (56,57,80). Thus, placental microvasculature and IH share expression of several placenta specific molecular markers and genetic profile, which led investigators to the pathogenetic hypothesis that placental damage during early gestation results in embolization of ectopic placental cells to fetal cutaneous vessels (55,56). A procedure of chorionic villous sampling, usually performed at 11 weeks of gestation, is believed to cause disruption of the placenta and possible embolization of placental cells (55). Higher risk for IH appears to be limited to the trans-cervically performed procedure, a finding that also suggests that precursor cells of IH origin from the placenta (53,55). Molecular genetic studies demonstrated no maternal-fetal microchimerism in children with IH confirming that the IH cells originate from the fetal part of the placental tissue (81,82).

Recent studies on the metastatic phenomenon of malignant tumors additionally support the idea of placental emboli in fetal circulation and from this angle placenta could be considered as a “tumor” and IH as a “metastasis” (83). Several case reports have described a correlation between the presence of CA and the incidence of neonatal hemangiomatosis (83). This correlation could support the hypothesis of the metastatic niche theory, which proposes that CA or placenta itself, secretes substances that prepare the implantation site, where IH occur; and that the IH precursor cells come from the placenta as a “benign metastasis” (83,84). First, in the preparation of the implantation site, fibronectin is laid down as a consequence of interaction between tumor cells and stromal cells (55,83). Thereafter, cell surface glycoproteins (CD 133, CD 34, CD 117), VLA4 and VEGFR1- expressing stem cells from the bone marrow arrive to the metastatic site (55,83). The next event, once the implantation site is complete, is arrival of tumor cells and the expression of VEGFR2 by precursor endothelial cells, which leads to angio-and vasculogenesis; expression of VEGFR1 is suppressed (55,83). Thus, the metastatic niche to which the tumor cells migrate is prepared and ready for tumor proliferation (55). In the case of a possible placental or CA origin of the

IH, the fetal side of the placenta or CA would be the source of hemangioma precursor cells secreting substances that prepare a site for IH, similar to a metastatic niche (55,83).

Progenitor cell theory suggests that a common origin of IH and placenta from circulating multipotent progenitor cells may explain the link between these two entities (54,55,56). The theory holds that IH derive from a somatic mutation in a primitive stem cell, which causes further development of these cells towards placental endothelial cells (55,56). It has been detected that endothelial progenitor cells express CD133 and CD34, cell surface glycoproteins, which were described also in metastatic niche theory as factors helping to prepare the metastatic site (55). This finding supports the hypothesis that endothelial progenitor cells may play an important role in the development of IH, possibly as precursors of the clonal endothelial cells (54,55). Peripheral blood analysis of IH patients has demonstrated that endothelial progenitor cells express placenta specific molecular markers such as GLUT-1, Fcγ RII and merosin, suggesting that endothelial progenitor cells may contribute to the rapid growth of IH (54,55).

1.4 CA AND IH IN PREECLAMPSIA AND MULTIPLE PREGNANCIES

Multiple gestations have 2-3 times higher incidence of preeclampsia, which is defined as gestation related hypertension with proteinuria, compared with singleton gestations (85,86,87,88,88,89). Also, the incidence of CA is 2-3 times higher in placentas from multiple pregnancies compared with the singleton placentas (12). Poor placentation due to abnormally limited superficial decidual cytotrophoblast invasion in the maternal spiral arteries supplying the placenta is the first stage in development of preeclampsia (91,92). Consequently, the transformation of spiral arteries to high caliber vessels is incomplete, as the myometrial segment of the vessels remain narrow, causing hypoperfusion of the placenta (91,92). The second stage in development of pre-eclampsia consists of repeated episodes of placental hypoxia, leading to an increased production of hypoxia inducible factor-1α (HIF-1α) and transforming growth factor-β1 (TGF-β1), increasing the secretion of a splice variant of the VEGF receptor sFlt-1 (soluble fms-like tyrosine kinase-1) (91). Recent studies suggest that maternal endothelial dysfunction resulting from placental soluble fms-like tyrosine kinase-1(sFlt1) and soluble endoglin (s-endoglin), two circulating antagonists to vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) may be part of a pathogenetic pathway in preeclampsia (6,86,90,93,94,95). Women with multiple pregnancies have demonstrated elevated levels of circulating sFlt1, s-endoglin and PlGF, and elevated sFlt1 to PlGF ratio compared with women with singleton pregnancies (93,94,96), these factors have also been discussed as pathogenetic inducers of CA. However, not all women with high sFlt1 and sFlt1 to PlGF ratio develop preeclampsia, which supports the hypothesis that preeclampsia has multifactorial pathogenesis and latter pathway is just one part of the complicated mechanism (93,94,95). On the contrary, cigarette smoking is associated with lower levels of sFlt1 and decreases the risk for preeclampsia (94,92). Two theories to explain the mechanism that leads to a cascade of events to develop preeclampsia in multiple

pregnancies have been proposed: 1. Placental hypoperfusion with intrauterine hypoxia, 2. Larger placental mass producing more preeclampsia related angiogenic factors (85,94). Hypoxia due to reduced placental perfusion has been demonstrated to induce sFlt1 production in vitro and in an animal model (85,93). On the other hand, a recent study failed to demonstrate correlation between larger placental mass and elevated anti-angiogenic profile in multiple compared with singleton pregnancies (93,97). In women with history of preeclampsia the subtle anti-angiogenic balance persists, which may explain the association of preeclampsia with an increased risk of cardiovascular diseases (93,97). It is unknown if elevated anti-angiogenic profile persists also in women with history of multiple pregnancies and if that could cause increased risk for cardiovascular diseases in this group as well (93). It has been speculated even that anti-angiogenic response may reduce maternal risk for breast cancer after multiple pregnancy and preeclampsia (93).

Clinically, gestational hypertensive disorders including preeclampsia demonstrate much more favorable neonatal outcome in multiple compared with singleton gestations (98). An explanation to this could be that elevated blood pressure in multiple pregnancies is may be a physiologic response to increased demand for blood (oxygen) supply by multiple fetuses (98). Accordingly, authors have been speculating that placentas from singleton pregnancies may show more pathological changes related to affected blood supply and hypoxia compared with placentas from multiple pregnancies, but there are no published studies describing these placental changes (98).

Multiple gestation is considered as a risk factor for increased incidence of IH in several studies (61,99). Multiple pregnancy may feasibly cause physical restriction of the placental development and poor growth of all fetuses may reflect a general uteroplacental dysfunction (100). On the other hand, discordant fetal growth in multiple pregnancies may be result of several factors including genetic differences between fetuses, dysfunction of one placenta and unequally shared placenta (100). The growth discordance in multiple pregnancies is defined as birth weight difference >25% between fetuses (100). In multiple pregnancies, generally smaller or sicker neonate is in bigger risk to develop IH. Greco et al, however, found that in many discordant twin pairs the larger twin developed IH, which indicates that there are other factors than low birth weight playing a role in pathogenesis of IH (99).

Furthermore, preeclampsia has been described as a prenatal factor possibly associated with development of IH (53). However, a recent study by Auger et al failed to demonstrate any direct influence of preeclampsia on development of IH (101).

2 AIMS

The overall aim of this thesis was to elucidate the pathogenesis of CAs, more specifically whether they are hypoxia related tumor-like-lesions or true tumors and find possible differences in the pathogenesis between large and small lesions. Further, to investigate the clinical significance of small CAs and assess whether the hypoxic background may be the common link between CAs and IHs.

The specific aim of each study was:

STUDY I

To study morphological characteristics of CAs and to analyze associations between CAs and hypoxia related morphological changes of placenta in singleton and multiple pregnancies.

STUDY II

To investigate clinical pregnancy characteristics and neonatal outcome in singleton and multiple pregnancies with CA and to explore the clinical significance of these lesions.

STUDY III

To examine a possible genetic background of large CAs by investigating large structural changes involving gain or loss of genetic material (deletions, duplications) by using array comparative genomic hybridization (a-CGH) method.

STUDY IV

To explore the epidemiological co-existence of CA and IH and their risk factors in singleton and multiple pregnancies.

3 MATERIALS AND METHODS

An illustrative flow chart of populations for all studies (study I-IV) is presented in Figure 3.

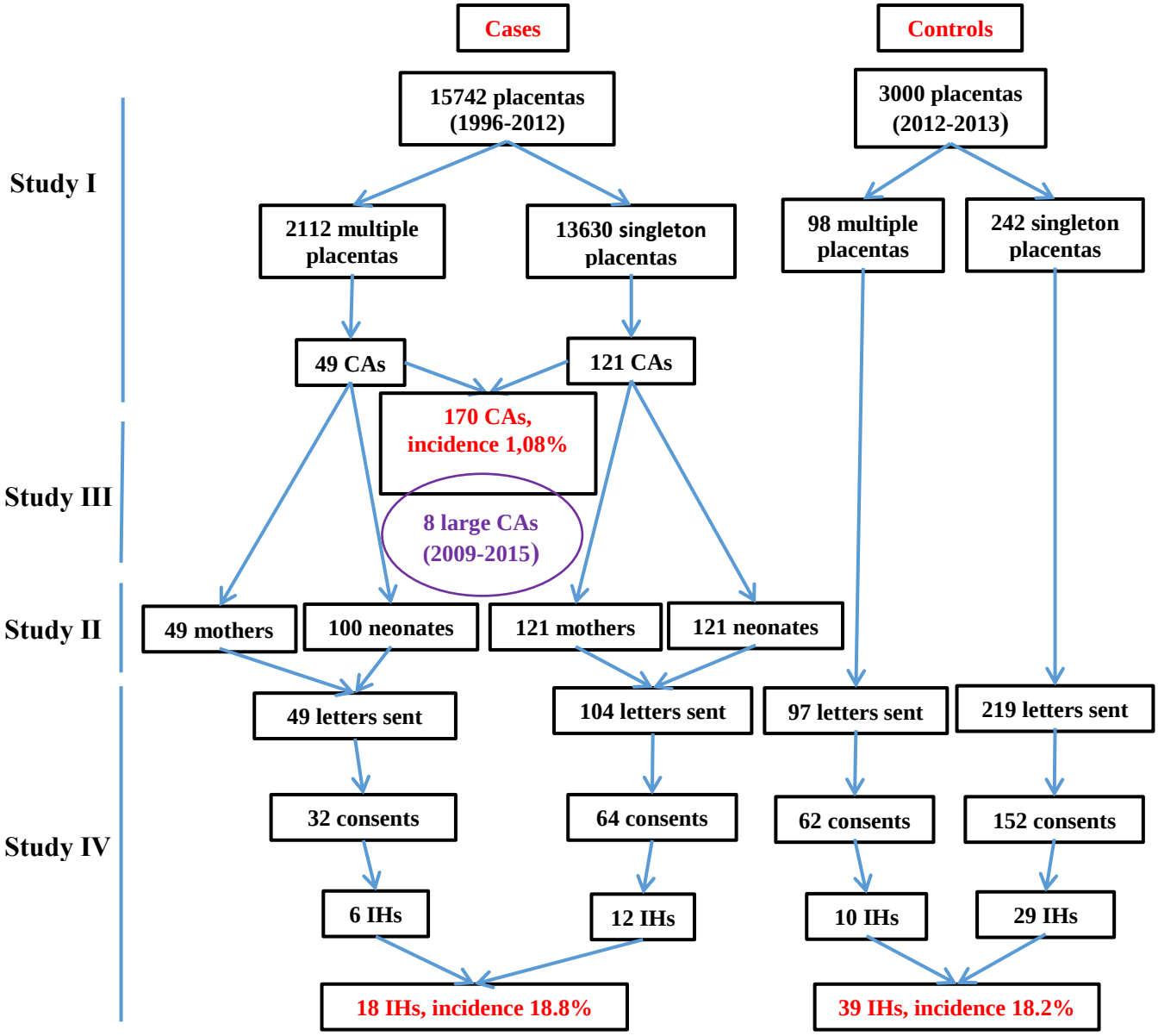

Figure 3. Flow chart of population for all four studies.

3.1 IDENTIFICATION OF CASES WITH CA AND CONTROLS WITHOUT CA

During the period of 1996-2012 15742 placentas, including 2112 (13,4%) from multiple pregnancies (2095 twin placentas and 17 triplet placentas), were examined at the Section for Perinatal Pathology, Department of Pathology at Karolinska University Hospital, Sweden. Placentas were referred for pathological examination according to the regional consensus indications and were morphologically examined based on a standardized, detailed protocol.

CAs were defined as nodular lesions composed of capillary vascular channels surrounded by trophoblast (3). One hundred and seventy CAs (from 121 singletons and 49 multiple

pregnancies) were identified during the study period, coded as hemangiomas according to the Systematized Nomenclature of Medicine (SNOMED). Gross examination data were collected from original reports and histological slides were re-reviewed by two perinatal pathologists.

Controls (242 singleton and 98 multiple placentas) were randomly selected from a cohort of 3000 placentas without CAs that were referred for morphological examination according to the regional consensus indications during the period of 2012-2013 (Figure 3). Controls were matched with the cases by the gestational age group and by the outcome of the pregnancy. Gestational age was retrieved from medical records and evaluated according to ultrasound examinations performed in the beginning of the second trimester.

3.2 PLACENTAL PATHOLOGICAL CHANGES

The histological variables that have been evaluated in all placentas were villous maturation, presence of decidual arteriopathy, infarction, intervillous thrombosis, abruption, fetal thrombi, chorioamnionitis and villitis; also, length, insertion site, coiling index and number of vessels of the umbilical cord. Morphological criteria for placental pathological changes are presented in Table 1.

CAs were measured and localization, morphological form and presence of multiple or single lesions were assessed. Placental weight adequacy to gestational age was assessed according to the tables by Pinar H *et al* (102,103).

Table 1. Morphological criteria for placental pathological changes.

Diagnosis	Morphological criteria
Maturation	Subjective assessment of delayed or accelerated maturation of chorionic villi compared with the gestational age
Infarction	Ischemic necrosis of chorionic villi of any size or localization
Decidual arteriopathy	Fibrinoid necrosis of maternal vessels with or without acute atherosis
Chorioamnionitis	Acute subchorionitis, chorionitis and/or chorioamnionitis
Vasculitis	Acute inflammation of vessels in chorionic plate and/or umbilical cord
Villitis	Foci of chronic villitis in any size
Intervillous thrombosis	Thrombosis of intervillous space, subchorionic thrombosis was not included
Umbilical cord insertion	Insertion up to 1 cm from the edge of the placenta or directly to the membranes was defined as the marginal/velamentous
Umbilical cord coiling index	Normal 0,07-0,30; coiling index was not calculated for umbilical cord shorter than 20 cm or for formalin-fixed placentas
Abruption	Retroplacental hematoma
Fetal thrombosis	Thrombosis in chorionic plate and/or stem villous vessels or umbilical vessels

3.3 MATERNAL CLINICAL CHARACTERISTICS AND NEONATAL OUTCOME

Maternal baseline characteristics, pregnancy and neonatal outcome data were retrieved from original medical records or electronic databases (Obstetrix, TakeCare) for cases with CA.

Neonatal birth weight adequacy to gestational age was evaluated according to Swedish growth charts (mean ± 2SD). The umbilical artery pulsatility index (PI) was calculated automatically by the ultrasound systems according to the method of Gosling et al. (5,104). Normal fetal umbilical artery blood flow velocity was classified as BFC 0 and abnormal as BFC I–III (105,106,107). Criteria for clinical characteristics are presented in Table 2.

Table 2. Criteria for clinical characteristics.

Clinical characteristics	Criteria
Maternal body mass index (BMI)	≥ 25 was considered as overweight
Preeclampsia	Systolic blood pressure ≥140 mmHg or diastolic ≥90 mmHg with or without proteinuria; HELLP
Diabetes	Fasting plasma glucose ≥ 7.0 mmol/l; Plasma glucose ≥ 11.1 mmol/l 2 hours after glycose tolerance test
Prematurity	delivery < 37th week of gestation
Apgar score at 5 min	<7 at 5 min was considered as low
Pulsatility index (PI)	PI > mean + 2 SD was classified as abnormal
Blood flow classes (BFC)	BFC 0 – positive diastolic flow, PI ≤ mean + 2 SD; BFC I - positive diastolic flow, PI > mean + 2 SD and PI ≤ mean + 3 SD; BFC II - positive diastolic flow, PI > mean + 3 SD and/or absent end diastolic flow; BFC III – absent end diastolic flow and/or reversed diastolic flow

3.4 GENETIC ANALYSIS OF LARGE CA

During the period of 2009-2016 fresh frozen tissue from eight large histological confirmed CAs and from unaffected tissue of same placenta was collected at the Pathology Department of Karolinska University Hospital, Stockholm, Sweden. All placentas were from singleton pregnancies, the gestational age varied from 28 to 41 weeks (mean 34.17 ± 4.71) and none of the cases had clinically diagnosed preeclampsia. The diameter of the lesions was in the range of 4 to 15 cm (mean 8.44 ± 3.7), two CAs showed histologically extensive necrotic and two partial degenerative changes. One case was from cohort of 170 CAs described above (Figure 3).

The genetic study consisted of array comparative genomic hybridization (a-CGH) using the Agilent 2x400K high-definition comparative genomic hybridization microarray (Agilent Technologies), including a total of 400000 oligonucleotide probes. The tissue from eight large CAs was analyzed in comparison with DNA from unaffected placenta tissue and pooled standard control genomic DNA (Promega, Madison, WI, USA), respectively, looking for

large unbalanced structural genomic changes (deletions and duplications). The pooled control genomic DNA consists of approximately 100 healthy individuals' DNA (not placenta). Comparison with normal placental tissue from the same individual excludes somatic chromosomal structural changes and with standard genomic DNA helps us to exclude constitutional large structural changes.

3.5 IDENTIFICATION OF IH IN CASES AND CONTROLS

An informative questionnaire with illustrative photos of IH was sent to 469 mothers (153 cases with CA and 316 controls) of 323 singleton (104 cases and 219 controls) and 146 multiple (49 cases and 97 controls) live born neonates registered in Sweden; individuals, which were extracted from the same cohort of 170 cases with CA and 340 controls without CA (Figure 3.). In total, 323 (68.9%) answers were received, of which 13 parents (4 cases and 9 controls) chose to be excluded from the study. Thus, in total 310 individuals (66.1%) provided their consent and were included to the study. The distribution between cases and controls was 96 vs. 214, respectively, and between singleton and multiple pregnancies was 216 vs. 94, respectively.

3.6 STATISTICAL ANALYSIS

In **study I**, CA characteristics and placental morphological changes of 170 placentas with CAs compared with the 340 matched controls were analyzed in two groups; placentas from singleton and multiple pregnancies; and were compared with the respective control group. In **study II**, maternal characteristics of 170 mothers with CA and neonatal outcomes of related 221 infants (121 from singleton pregnancy and 100 from multiple pregnancies) were analyzed comparingly in singleton and multiple pregnancies; control groups were not included. Further, in both studies the characteristics were analyzed in association to the diameter of CA.

In **study IV**, 310 patients (96 cases and 214 controls) of 216 singleton (64 cases and 152 controls) and 94 multiple (32 cases and 62 controls) pregnancies were analyzed accordingly: singleton compared with multiple gestation, and cases with CA compared with controls.

Statistical analysis for all studies was performed using the R software version 3.0.3 (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/)

The following tests were used where appropriate: The Pearson's chi-squared and the Fisher's exact (in case of small sample size) tests were applied to examine the relationship of categorical data on contingency table analysis. The Shapiro-Wilk normality test was used to determine whether sample data has been normally distributed. The non-parametric tests, Wilcoxon rank-sum and Kruskal-Wallis (in variables with more than two groups) applied in the analysis of variables, which were non-normally distributed. Uni- or

multivariate logistic regression analysis of data applied to estimate odds ratio (OR) values with 95% confidence intervals (CI). A p value of <0.05 was considered significant for all analyses.

Study III consisted of two parallel comparison analyses of CA with different controls. One analysis found no differences between two compared groups and second analysis revealed altogether 48 common CNVs. Thus, statistical analysis was not applicable.

3.7 ETHICAL CONSIDERATIONS AND PERMISSIONS

The placenta tissue material (paraffin embedded blocks and histological slides) is stored in the Department of Pathology according to the Swedish Act on Biobanks (2002:297), and is based on patients` informed consent. Ethical permissions are required for using this tissue material in research according to the Act concerning the Ethical Review of Research involving Humans (2003:460).

All studies were approved by the Regional Ethical Review Board in Stockholm, Sweden.

Dnr: 2012/1430-31/1 approved 2012-10-16 (Study I-IV).

Dnr: 2014/158-32 approved 2014-03-25 (Study I, III).

4 RESULTS

4.1 CA CHARACTERISTICS

The overall incidence of CAs was 1.08% in our cohort of 15742 placentas. **Study I** demonstrated that the frequency of CA was significantly higher in the group of multiple compared with the singleton pregnancies (2.32% vs 0.89%, p<0.0001). Most of the tumors showed nodular structure (n=144, 84.70%), were presented as single lesions (n=140, 82.35%), located subchorially (n=122, 71.76%) and discovered already during the gross examination of the placenta (n=95, 55.88%). Overall, singleton and multiple placenta groups showed no statistically significant differences in morphological characteristics of CAs (Figure 4).

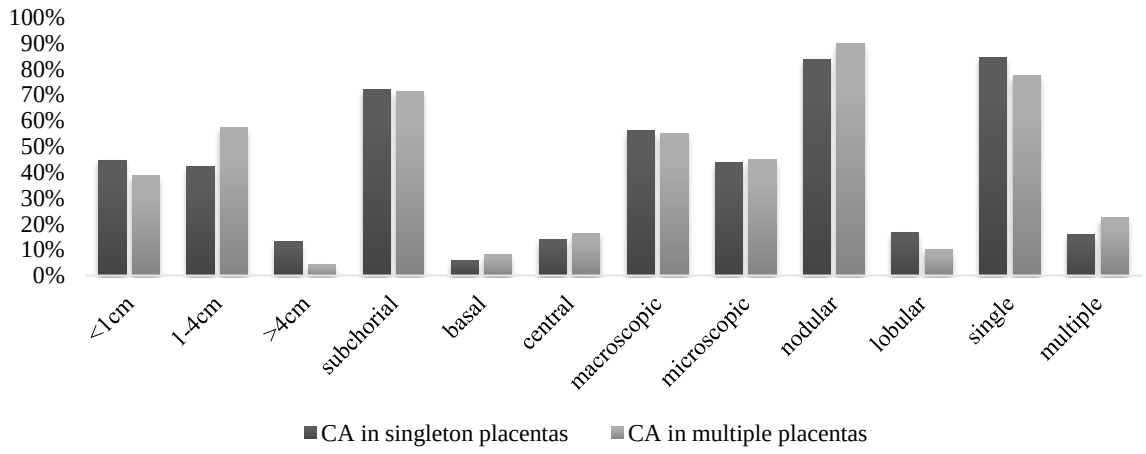

Figure 4. Morphological characteristics of CAs in singleton and multiple placentas.

The diameter of CA ranged from 0.05 cm to 13.5 cm, median (IQR) 1.1 cm (0.5-1.9). The smaller median diameter of the CA was associated with small for gestational age (SGA) placentas, accelerated chorionic villous maturation, placenta infarctions, decidual arteriopathy and absence of fetal thrombosis. **Study II** showed that in singleton pregnancies the smaller median diameter of the CA was related to preeclampsia, whereas multiparity and maternal smoking were associated to a larger median diameter of CA. In neonates from singleton pregnancies the smaller median diameter of CAs was found in SGA neonates, as well as in the group of neonates admitted to the neonatal care unit. On the other hand, in neonates from multiple pregnancies no statistical significant associations between any of the studied neonatal outcomes and the diameter of the CAs were observed. In **study IV**, the diameter of CA in cases of co-existence of CA and IH (n=18) ranged from 0.1 to 10 cm, the median (IQR) 1.25 cm (0.78-2.48); indicating that the size of CA was not related to the development of IH.

Array-CGH analysis of eight large CAs in **study III** detected altogether 48 common polymorphic CNVs in eight CA samples (median=5.5 per sample) when compared with pooled standard control DNA and none when compared with DNA from unaffected placenta from the same individual. All described structural changes (duplications and deletions) were found in databases (Database of Genomic Variants, http://dgv.tcag.ca/dgv/app/home) of known polymorphisms and classified as benign. No common pathogenic, rare or novel structural changes involving gain or loss of genetic material compared with either standard control DNA or unaffected placenta DNA were revealed.

4.2 PLACENTAL MORPHOLOGICAL CHARACTERISTICS

One hundred and seventy placentas with CA included 49 multiple placentas of which 29 were dichorionic, 18 monochorionic and 2 trichorionic placentas.

In singleton placentas with CAs the incidence of infarction (p=0.0000055**)**, decidual arteriopathy (p=0.0004), fetal thrombosis (p=0.000035), hypercoiled umbilical cords (p=0.0004), large for gestational age (LGA) placentas (p=0.00011), accelerated (p=0.00059) and delayed maturation of chorionic villi (p=0.04) was significantly higher compared with the control group (Figure 5).

Multiple placentas with CAs showed significantly higher incidence of fetal thrombosis (16.33% vs 4.08%, OR=4.58, p=0.017) in comparison with the control group (Figure 5). None of the other assessed variables reach the level of statistical significance.

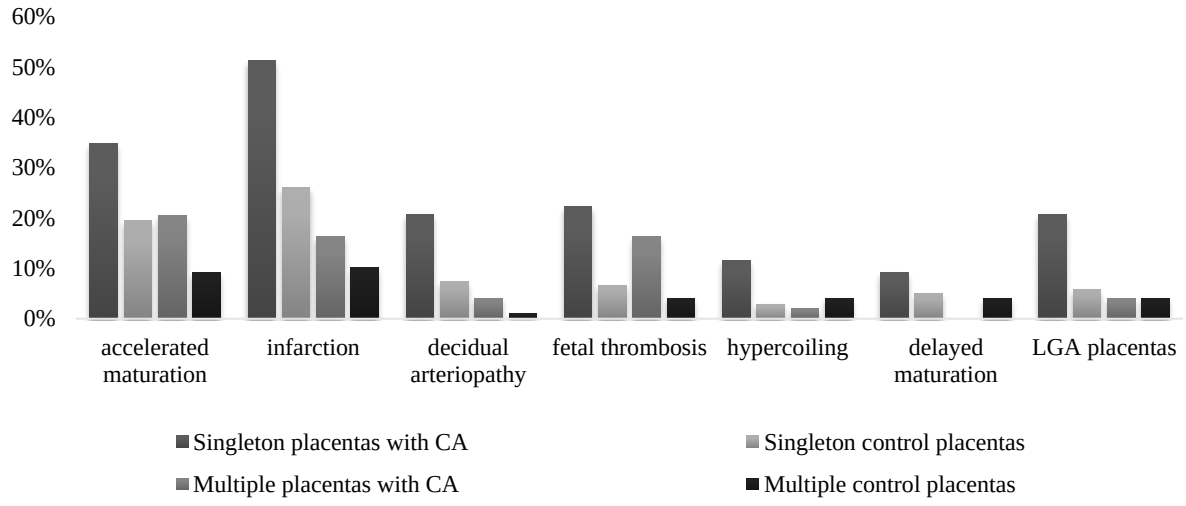

Figure 5. Incidence of hypoxia-related placental changes with statistically significant differences in multiple and singleton placentas with CAs compared with the controls.

4.3 MATERNAL BASELINE CHARACTERISTICS

The gestational age in cases with CAs (n=170) ranged from 20 to 43 weeks, the median (IQR) gestational age was 37 weeks (33.25-39); the median (IQR) in singleton pregnancies was 36 (33-39) weeks and in multiple pregnancies was 37 (35-38) weeks. The frequency distribution between gestational age groups in singleton and multiple pregnancies is presented in Figure 6.

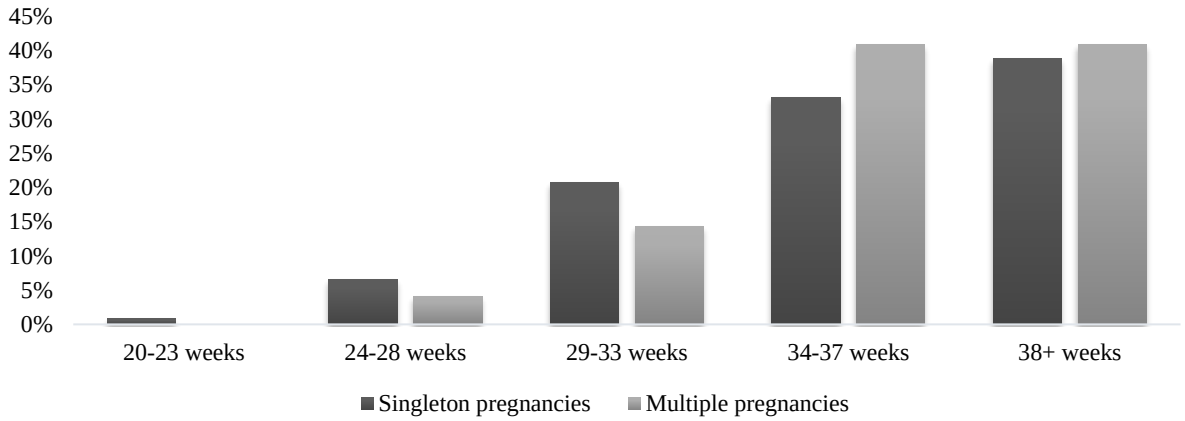

Figure 6. The frequency distribution between gestational age groups in singleton and multiple pregnancies.

Maternal baseline characteristics in singleton and multiple pregnancies demonstrated no statistically significant differences between the two studied groups. Maternal age (mean ± SD) was 31.3 ± 5.7 years in singleton pregnancies and 33 ± 4.9 years in multiple pregnancies. The distribution of maternal baseline characteristics between the studied groups is presented in Figure 7.

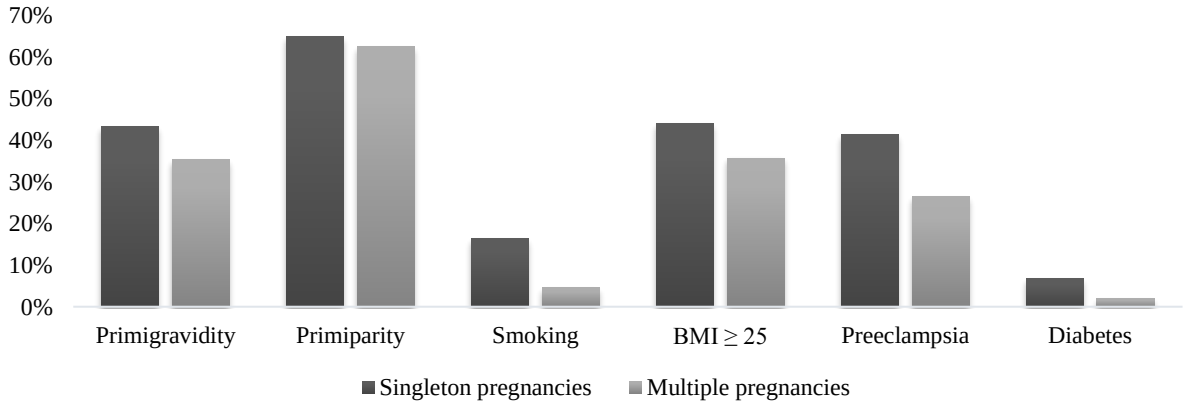

Figure 7. Distribution of maternal baseline characteristics in singleton and multiple pregnancies.

4.4 NEONATAL OUTCOME

Neonates from singleton compared with multiple pregnancies demonstrated significantly more adverse outcome including higher incidence of low 5-minute Apgar score (11.02% vs 4.04%, OR=3.21, p=0.047), abnormal PI (33.33% vs 16.67%, OR=2.50, p=0.037) and BFC ≥ 1 (33.96% vs 14.49%, OR=3.03, p=0.013). Furthermore, the frequency of stillbirths (10.13% vs 3.03%, p=0.057) was also higher in singleton compared with the multiple neonates. The incidence of CA was higher in female (n=122, 55.20%) compared with male (n=94, 42.53%) neonates. The overview of neonatal outcome characteristics in multiple and singleton pregnancies is presented in the Figure 8.

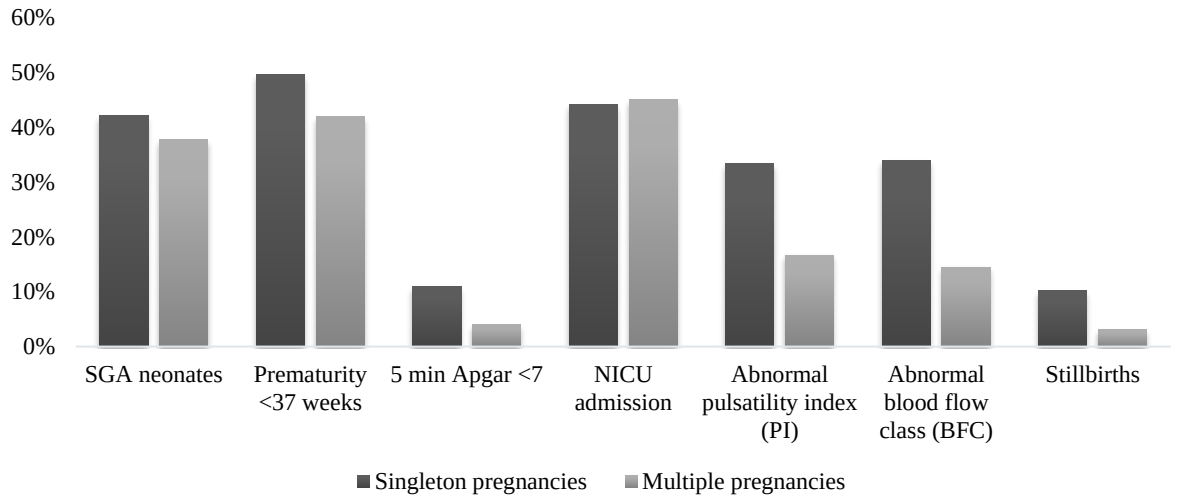

Figure 8. Neonatal outcome characteristics in singleton and multiple pregnancies.

4.5 INCIDENCE OF IH

The incidence of IH showed no statistically significant differences either between cases with CA and controls or between singleton and multiple pregnancies. Overall, 57 cases of IH were registered in the cohort of 310 individuals.

The incidence of IH was higher in female (n=33, 60%) compared with male (n=22, 40%) neonates and more females were affected in singleton (n=25, 60.9%) compared with multiple (n=8, 57.1%) pregnancies; and in controls (n=23, 62.1%) compared with cases (n=10, 55.6%). However, none of these differences reached the level of statistical significance.

The incidence of preeclampsia in our selected, high-risk pregnancy cohort demonstrated statistically significant differences between the studied groups. A higher incidence of preeclampsia was found in cases compared with controls (n=40, 41.7% vs n=52, 24.3%, p=0.002) and in singleton compared with multiple pregnancies (n=72, 33.3% vs n=20, 21.3%, p=0.034). However, the incidence of IH was not associated with preeclampsia, since there was no statistically significant difference between patients with (n=22, 23.9%) and without preeclampsia (n=35, 16.1%).

Further, SGA neonates were significantly more common in cases compared with controls (n=51, 53.1% vs n=64, 30.1%, p=0.00012), and in multiple compared with singleton pregnancies (n=44, 46.8% vs n=71, 33.0%, p=0.021).

However, none of the studied characteristics demonstrated statistically significant differences in relation to the incidence of IH either between cases with CA and controls or between singleton and multiple pregnancies.

Multivariate logistic regression analysis showed significantly higher incidence of CA in SGA neonates and in cases with preeclampsia. However, prematurity was significantly higher in controls suggesting that it may have a protective impact on incidence of CAs in our cohort (Table 5). On the other hand, prematurity was the only characteristic associated with higher incidence of IH (Table 6).

Table 5. Multivariate logistic regression analysis of risk factors for the development of CA.

Variables[1]	with CA (N,%)	without CA (N, %)	OR(95%CI) [2]	*P value*
SGA[3]	**51(53.1)**	**64(30.1)**	**2.08(1.19 -3.63)**	**0.010**
Gender(females)	52(54.2)	96(44.9)	1.20(0.70-2.04)	0.495
Prematurity(yes)	**42(43.8)**	**117(54.7)**	**0.51(0.29-0.90)**	**0.021**
Preeclampsia(yes)	**40(41.7)**	**52(24.3)**	**2.09(1.15-3.79)**	**0.015**
Pregnancies(multiple)	32(33.3)	62(29.0)	1.36(0.74-2.47)	0.311

Bold font indicates significant results ($p<0.05$); [1]The references for variables entered into the model were as follows: SGA (No SGA); Gender (Males); Prematurity (No Prematurity); Preeclampsia (No Preeclampsia); Pregnancies (Single). [2]Odds ratio (OR) values with 95% confidence intervals (CI); [3]SGA- small for gestational age.

Table 6. Multivariate logistic regression analysis of risk factors for the development of IH.

Variables[1]	with IH (N,%)	without IH (N, %)	OR(95%CI) [2]	*P value*
SGA[3]	20(35.1)	95(37.7)	0.77(0.38-1.56)	0.484
Gender(females)	33(60.0)	115(50.4)	1.51(0.81-2.81)	0.187
Prematurity(yes)	**42(73.7)**	**117(46.2)**	**3.18(1.61-6.25)**	**0.00078**
Preeclampsia(yes)	22(38.6)	70(27.7)	1.40(0.70-2.76)	0.330
Pregnancies(multiple)	16(28.1)	78(30.8)	1.22(0.59-2.52)	0.588
Cases(CAs)	18(31.6)	78(30.8)	1.16(0.58-2.30)	0.664

Bold font indicates significant results ($p<0.05$); [1]The references for variables entered into the model were as follows: SGA (No SGA); Gender (Males); Prematurity (No Prematurity); Preeclampsia (No Preeclampsia); Pregnancies (Single); Controls (without CAs). [2]Odds ratio (OR) values with 95% confidence intervals (CI); [3]SGA- small for gestational age.

5 DISCUSSION

5.1 MAIN FINDINGS OF THE STUDY

The incidence of CA in our population of examined placentas from high-risk pregnancies was 1.08%, which is in line with previously published data.

Placentas from multiple pregnancies in our study cohort demonstrated higher incidence of CAs compared with singleton placentas, which is in accordance with previous studies.

Placentas from singleton pregnancies with CAs were more affected by hypoxia associated morphological changes compared with placentas from multiple pregnancies. Consequently, neonatal outcome characteristics followed the same pattern demonstrating an increased rate of adverse neonatal outcome in singleton compared with multiple pregnancies.

Smaller diameter of CA was related to the higher risk of placental affection by hypoxia associated morphological changes, preeclampsia and less favorable neonatal outcome. Small CAs are more likely hypoxic vascular hyperplasias associated with malperfusion of the placenta, rather than true tumors.

Genetic analysis of eight large CAs showed no pathogenic structural abnormalities (deletions or duplications) in the genome and no structural genomic variation differences between CA and unaffected placenta.

The incidence of IH in our study cohort was 18%, which is higher than previously reported. However, no association between CA, gender, preeclampsia, multiple gestation or small for gestational age neonates and increased risk of IH was found.

5.2 INTERPRETATION OF FINDINGS

5.2.1 CA association to hypoxia

CA is a well circumscribed nodular villous capillary lesion of the placenta occurring more frequently in pregnancies at high altitude and in association to preeclampsia and multiple gestation (3). Thus, CAs are thought to be hypoxia-associated capillary proliferations mediated by several growth factors and related to adverse gestational and environmental influences, rather than to be true tumors (1,3). Noteworthy, hypoxia has been reported to be the major pathophysiologic factor to control angiogenesis (75).

Hypoxia-associated placental pathological changes can be caused by several different mechanisms including: decreased oxygen pressure at high altitude with secondary maternal hypoxemia, maternal anemia with reduced oxygen carrying capacity of the blood, maternal smoking, overdistension of the uterus by multiple gestation and increased demand for oxygen by multiple fetuses and decidual arteriopathy with insufficient spiral artery remodeling and trophoblast invasion in preeclampsia (107,108). Furthermore, hypoxia-related morphological changes can be classified as focal (placental infarction, CA) or more global (accelerated maturation of chorionic villi). However, the placental hypoxia in clinically diagnosed preeclampsia is more likely a focal rather a global tissue injury depending on the number of affected maternal uterine arteries (108,109).

5.2.2 Placenta hypoxic changes and neonatal outcome in association to CA

In our tertiary institution, only highly selected placental material is referred for histopathological examination, according to the regional consensus indications. Therefore, all our studies (**study I-IV**) comprise a high-risk pregnancy population with a high incidence of preeclampsia (41.32% in singleton and 26.53 % in multiple pregnancies).

In **study I,** we have assessed overall fourteen different morphological hypoxia associated changes of the placenta, seven of which showed statistically significant differences between cases and controls in singleton placentas. Decidual arteriopathy, accelerated maturation of chorionic villi and infarctions are the morphological indicators of the uteroplacental malperfusion and often related to the clinically diagnosed preeclampsia, reflecting a decreased intake of oxygen from the intervillous space (108). On the other hand, large for gestational age placentas and delayed maturation of chorionic villi, which were also associated with an increased risk for CAs in singleton placentas, are morphological indications for maternal diabetes mellitus, a status related to intrauterine hypoxia as well (108). Moreover, villous hypomaturity has been reported to be associated with increased risk of fetal fatality (3,108). Hypercoiling of umbilical cord has been described in relation to delayed maturation of chorionic villi and reduced number of villous vasculosyncytial membranes, which are morphological features for placental hypoxia (108). In addition, abnormal coiling of the umbilical cord may be related to vascular thrombosis in the chorionic

plate (110). The frequency of fetal thrombosis in **study I** was significantly higher in both the singleton and multiple placentas with CAs compared with the controls. Further analysis revealed that the incidence of fetal thrombosis was associated with larger diameter of CAs, which may be explained by the mechanical compression of the vessels in the chorionic plate by the larger tumors, thus fetal thrombosis could be secondary to large CA. Moreover, Das et al have speculated that large CAs could even produce prothrombotic factors resulting in fetal thrombosis (111).

Based on **study I**, smaller diameter of CAs was associated with hypoxia related placental changes indicating malperfusion of the placenta. Similarly, in **study II** preeclampsia and small for gestational age (SGA) neonates in singleton pregnancies were associated to smaller CAs. Accordingly, we hypothesized that small CAs could be considered as hypoxic vascular hyperplasias induced often by preeclampsia rather than true tumors. Hence, the recognition of CAs as potential morphologic indicator of placental hypoxia and/or preeclampsia could be a valuable information for clinical placental diagnostics.

In **study I**, placentas from multiple pregnancies with CAs were less affected by hypoxia associated changes than singleton placentas with CAs compared with controls. Accordingly, we speculated that the low incidence of hypoxia-associated placental changes in multiple pregnancies could be explained by the circumstance that multiple pregnancies *per se* with increased demand for oxygen from multiple fetuses are associated with better adaptation to the placental tissue hypoxia. This speculation may be further supported by our observation from **study II** that in singleton pregnancies CAs seem to be associated with an increased distal placental resistance showing abnormal blood flow velocity waveforms in the umbilical artery, while in multiple pregnancies such phenomenon is less common.

In **study II**, neonates from multiple pregnancies with CAs demonstrated a much more favorable outcome compared with neonates from singleton pregnancies with CAs. Accordingly, they showed lower incidence of low 5-minute Apgar score, stillbirths, pathological PI and BFC ≥1. The latter finding follows the same pattern as described in our **study I**. Furthermore, the frequency of preeclampsia was very high in our high-risk pregnancy cohort and it has been suggested that due to the increased demand for oxygen and nutrients, pregnancy induced hypertensive disorders, including preeclampsia might be related to a better fetal blood supply in multiple pregnancies (98,112). Consequently, hypertensive disorders of pregnancy might have protective effect against low 5-minute Apgar score and might be beneficial for fetal survival in multiple compared with singleton gravidities (98,112).

Noteworthy, placental hypoperfusion resulting to intrauterine hypoxia elevates the levels of circulating angiogenic/antiangiogenic factors of placental origin including the soluble fms-like tyrosine kinase 1 (sFlt-1) and sFlt-1/PlGF ratio (93). Similar alterations in serum levels of these factors have been demonstrated in preeclampsia and in multiple pregnancies (86,93). Our speculation of placental tissue hypoxia in multiple pregnancies could explain the increased level of sFlt-1 and sFlt-1/PlGF ratio in multiple compared with singleton

pregnancies without preeclampsia (86,93). The latter might suggest a shared pathogenetic mechanisms and/or to reflect an increased hypoxic environment *in utero* in multiple pregnancies as well as in preeclampsia (93).

5.2.3 Genetic background of CA

The genetic background of CAs is so far poorly elucidated. However, the occasional reports of recurrence of CA suggest that genetic factors may play role in the pathogenesis of these lesions (11,36,40). Only few case reports, including cytogenetic data of CA can be found in the literature and there are no systematic studies on this topic. We designed our **study III** as a pilot study of eight large CAs investigating large unbalanced genetic structural variants (deletions and duplications) using a-CGH method. The selected genotyping platform was not targeted specifically at genes within the angio/vasculogenetic pathways but included 180000 probes and covered the entire genome, as well as targeted 1989 specific genes including VEGFA, TGFβ family and PIGF.

Vascular anomalies including hemangiomas are a heterogenous group of lesions and causative mutations have been described in genes encoding proteins involved in the angiogenesis signaling pathways (113). Although the specific genetic etiology in most vascular anomalies is still unknown, the identification of putative genes related to these lesions, could provide a better understanding of their pathogenesis (113). Approximately 30 different genes have been reported in the literature so far in association with vascular anomalies including hemangiomas, which emphasizes that different genetic mutations can cause the same vascular anomaly (allelic heterogeneity), a single gene mutation can be related to more than one different vascular anomaly (phenotypic heterogeneity), and multiple genes can be associated with clinically similar vascular anomalies (locus heterogeneity) (113).

We investigated the possible genetic contribution to development of large CAs and failed to find any pathogenic large genetic changes (duplications and deletions) in our relatively small sample size of eight large CAs. This lack of genetic aberrations could support a non-tumorous genesis of these lesions. On the other hand, hypoxia-related origin of CAs might explain the lack of the presence of pathological genetic aberrations. However, additional genetic studies are required to exclude the genetic contribution to development of CAs and to investigate further possible hypoxia-related genesis of these lesions.

5.2.4 Placenta theory of IH pathogenesis

The specific etiology of IHs remains still unclear despite of many proposed etiopathogenetic mechanisms and hypotheses. The theory of placental origin is based on several similarities on the level of cell surface markers and transcriptome shared by endothelial cells of IH and

placental microvasculature (56,57,58,79). Accordingly, the placenta theory of pathogenetic mechanism of IH hypothesizes that possible placenta tissue damage during gestation results in embolization of ectopic placental endothelial cells to the fetal circulation and these cells start proliferating in the areas with hypoxic conditions (16,82). Tissue hypoxia is a powerful inducer of angio- and vasculogenesis through an increased expression of angiogenic factors including VEGF, PlGF, Flt-1 (a high-affinity transmembrane receptor for both PlGF and VEGF), and sFlt-1. Therefore, tissue hypoxic status or *in utero* hypoxia could act as an impulse to trigger the principal endothelial cells and initiate a cascade of reactions causing the proliferation of endothelial cells and subsequent formation of IH and/or CA (14,75,82).

5.2.5 Incidence of IH in association to CA

IH and CA share several risk factors, which are often related to hypoxic influence. An increased incidence of IH and CA has been reported in association with higher maternal age, preeclampsia, fetal female sex, prematurity and multiple gestation (3,14,61). In addition, higher risk for IH is related to low birth weight and Caucasian race (61).

Study IV did not demonstrate any significant correlations between CA, preeclampsia, SGA neonates or multiple gestations and increased risk of IH. Even multivariate logistic regression analysis revealed significantly higher risk of IH only in cases of prematurity. On the other hand, our study cohort consisted of high risk pregnancies and therefore results may not be generalized to the whole population. Female neonates in our cohort showed slightly higher incidence of developing IH compared with males, but the difference did not reach the level of statistical significance. The mechanism, by which female neonates are more often affected by IH is suggested to be associated with the higher levels of renin compared with male neonates (65). An increased level of renin induces a higher level of angiotensin II, which in turn is an activator of the endothelial cell proliferation (65). Same mechanism could explain an increased incidence of IH in Caucasians compared with Blacks, premature compared with full-term infants, and children compared with adults (65). Even the spontaneous involution of IH could be explained by the latter mechanism: the renin levels decrease, as children grow older (65). Nevertheless, our study was based on the informative questionnaire answered by the parents. Accordingly, we did not go through medical records of the individuals to confirm the diagnosis of IH clinically or to collected the information about renin levels for patients. Therefore, our study did not include information about renin levels of the individuals and possible associations between renin levels and studied characteristics.

5.3 METHODOLOGICAL CONSIDERATIONS

To the best of our knowledge, it is the largest cohort of CAs reported to the literature so far.

Retrospective design of **studies I, II** sets limits on a collection of the data as it is impossible to re-examine the placenta macroscopically or study patients according to the designed protocol. There is no possibility for additional clinical tests or complementary tissue samples from the placenta; investigator is dependent on existing data which is a major disadvantage of a retrospective study design.

Our study cohort originates from tertiary referral center and in our institution only selected placentas are referred for a morphological examination, according to the regional consensus indications. Therefore, the results of **study I** and **II** reflect high-risk pregnancies.

Recruitment of controls in placental material is always problematic as placental normal morphology is changing during gestational weeks, thus healthy term placentas would not reflect placental morphological changes in different developmental stage and would therefore be suboptimal in a role of controls. An ideal control group would contain healthy placentas from gestational age matched with cases; however, recruitment of such a control group is unachievable as cases in our study were ranged from gestational week 20 to 43. In **study I**, for morphological analysis, we decided to use control group matched with cases by gestational age group and pregnancy outcome. Controls without CA originate also from our selected placenta population. In **study II,** we decided not to use controls or reference material to compare since the study comprises a selected, high-risk pregnancy cohort with a high incidence of preeclampsia and therefore comparison with a normal reference group would have been of limited value. **Study II** was meticulously designed to assess the differences in clinical outcomes between multiple and singleton pregnancies with CA and to further elucidate the related neonatal outcomes. In **study IV** we decided to use our, by gestational age matched, controls and not to use healthy term controls as gestational age and prematurity were also important variables to analyze. Based on ethical considerations and predictable clinical course of IH only live born neonates were included to the study.

In **studies I** and **II** we have considered even distinguishing between the different types of multiple gestations (di/trichorionic and monochorionic) to analyze the influence of different vascular patterns in different types of placentas, despite a relatively small group of observations (n=49). However, the number of the observations in subgroups ended up very small and unequally distributed, thus further analysis might undermine the reliability of findings (positive or negative results). Consequently, we decided not to proceed further with latter analysis.

In statistical analysis, we have used median value, instead of mean value, and the inter-quartile range (IQR) is shown instead of the standard deviation (SD) for the diameter of CA, gestational age and BMI because the respective variables were determined to have non-normal distributions. Consequently, non-parametric tests (Wilcoxon rank-sum and

Kruskal-Wallis) were applied in our study to assess the relationship between the diameter of CAs and the studied characteristics, where appropriate.

For **study III,** fresh frozen tissue material was collected only from large CAs since from small or microscopic CA fresh frozen tissue cannot be collected as the diagnosis is mostly confirmed only after careful microscopic examination. Analysis of large CAs was expected to be challenging because of degenerative changes (i.e. necrosis, fibrosis, hyalinization and calcifications) of the tissue, which often are extensive in these lesions. However, the frozen tissue for genetic analysis was collected from macroscopically preserved areas of lesions. Based on results of our study, all analyzed CAs showed similar pattern of common CNVs. Accordingly, degenerative tissue changes did not play substantial role in the results of our analysis.

The limitation of **study III** was the relatively small sample size. Large CAs are rare lesions and to collect larger sample set would take additional years. Moreover, only one area from CAs and unaffected placenta was analyzed. In future, should be considered to collect multiple biopsies from both locations to cover all/as many as possible different tissue changes. Also, our chosen method (a-CGH) cannot detect smaller (<30 kb) genomic alterations and, inherently to the method, cannot identify balanced structural chromosomal changes and more likely fail to detect mosaicism. Therefore, additional genetic studies are required to assess the genetic contribution in genesis of CAs.

A high incidence of IH (18.4%) in **study IV** could be explained by a selection bias of our study design, which was based on an information from parents and not from clinical records. Accordingly, parents whose children had history of IH probably were more interested in the topic and for this reason more engaged to participate in the study. Furthermore, the incidence of IH might be also higher in our study than in medical records since it was reported directly by parents and not confirmed clinically; parents might have recorded more IH cases, even those, which did not require clinical attention. Thus, in our study parental memory has been considered as an advantage, to report more not less cases of IH.

6 CONCLUSIONS

Study I explored the association of CAs with several morphological placental changes in the largest cohort of CAs reported in the literature so far. The analysis of data from **study I** confirm that the incidence of CAs is 2.6 times higher in placentas from multiple compared with singleton pregnancies. Furthermore, we demonstrated that CAs were associated with an increased rate of hypoxia related placental changes in singleton placentas which supports the hypothesis of a hypoxia-driven origin of CAs.

Study II showed a much more favorable neonatal outcome in multiple compared with singleton pregnancies with placental CAs, which might reflect an increased demand of oxygen from multiple fetuses and a consequential adaptation to the placental hypoxia per se in this group. In addition, our finding of an increased rate of an adverse neonatal outcome in singleton compared with multiple pregnancies is in line with our results from **study I**.

The high incidence of preeclampsia in both singleton and multiple pregnancy groups, reflects a high-risk status of our study cohort. Contrary to the literature, preeclampsia was more common in our singleton rather to multiple pregnancy group. Remarkably, the high incidence (5-8 times higher than in general population) of preeclampsia in the cohort of CAs might lead us to identify these lesions as a potential morphologic indicator of placental hypoxia, which in turn could be a valuable information for morphologic placental diagnostics and in clinical approach of CAs.

Study III as a pilot study investigated the genetic background of CAs by analyzing structural variations (duplications and deletions) in the genome of eight large CAs. The current analysis failed to demonstrate any pathogenic chromosomal structural abnormalities in the CA genome and to reveal any genetic structural differences when compared with control placenta. This lack of findings could support a non-tumorous origin of these lesions, thus contribute indirectly to the hypothesis of hypoxia-driven genesis of CAs. However, additional genetic studies are required to fully explore the role of genetic factor in the development of CAs.

Study IV showed that in our cohort of 310 patients, the co-occurrence of CA and IH was present in 18 cases. However, we failed to confirm an increased incidence of IH in cases of CA, multiple pregnancies or preeclampsia described in the literature. According to **study IV**, an increased incidence of CA was related to the preeclampsia and small for gestational age birth weight, whereas prematurity was the only characteristic associated with higher incidence of IH. Consequently, the presence of CA in the placenta is not increasing the risk to develop IH, although the latter observation is not excluding the hypothesis of placental origin of IH.

7 FUTURE PERSPECTIVES

The specific etiology and clinical significance of CAs is still unclear and the research on CAs is mostly limited by case reports in the scientific literature. A better understanding of etiological factors of CAs would help us to evaluate more precisely also the clinical significance of these lesions, therefore more systematic studies in this field would be highly appreciated.

The current advance technology of using whole genome sequencing (WGS) could be a powerful tool to provide novel insights in the pathogenesis of CAs. Consequently, we have proceeded with genetic analysis of eight large CAs using the WGS method and this has given us a large amount of unique data to continue working with. WGS analysis provides us with the capability to discover smaller (<30 kb) structural variations in the human genome, including deletions, duplications and inversions; as well as to study single nucleotide variations. Of interest, we could proceed with pathway based gene analysis including, putative genes, which provide causal risk for vascular anomalies and genes that are related to hypoxia status.

In our clinical material of multiple placentas, which were perfused with colors for investigating vascular anastomosis we have noticed that CAs are apparently supplied in some cases by umbilical artery, whereas in other cases from umbilical vein circulation. As a future prospect, it would be interesting to investigate to which circulation the feeding vessels belong by perfusing the placentas containing CA with colors through umbilical vessels.

Our study IV was based only on an informative questionnaire, which was answered by parents we have not had access to medical records of patients to confirm the diagnosis of IH or collect clinical data. However, would be interesting to design a clinical prospective study of IH patients with clinically confirmed diagnosis and additional information of renin levels. The measurements of renin level are not included in the routine clinical approach of these patients; thus, the study should be with a prospective character.

In addition, the pathogenesis of CAs and IHs could be explored further by comparing the immunohistochemical expression of several hypoxia related or angiogenetic factors ((HIF-1α, TGF-β1, VEGF, PlGF, Flt-1 and sFlt-1) on paraffin embedded material of these two lesions. Furthermore, to study retrospectively, where possible, placentas of patients with IH in comparison with controls.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Bakgrund: Chorangiom (CA) är den vanligaste tumör-liknande förändring i moderkakan (placenta), men dess uppkomstmekanismer är ofullständigt klarlagda och eventuella genetiska faktorer är outforskade. CA består av fetala kapillärer och finns i c: a 0.5 – 1% av alla placentor. I placentan observeras de oftast inom de minst väl syresatta områden. Dessutom är CA vanligare i graviditeter på hög höjd samt i samband med havandeskapsförgiftning (preeklampsi). Dessa observationer har lett till hypotesen att CA är kärlproliferationer associerade till kronisk syrebrist (hypoxi) i placenta snarare än äkta tumörformer. CA kan vara små (<1cm) och upptäckts då oftast accidentellt vid patologisk undersökning av placenta. Alternativt är CA stora (> 5–8 cm) och kan i dessa fall upptäckas vid prenatal ultraljudsundersökning och eventuellt behandlas. De stora CA är förknippade med allvarliga graviditetskomplikationer, såsom fosterdöd, tillväxthämning, fetal hydrops mm. Infantilt hemangiom (IH) är den vanligaste tumörformen under barndom. IH är i likhet med CA kapillära proliferationer och karaktäriseras av ett till stor del förutsägbart naturalförlopp, där majoriteten genomgår spontan regress oftast innan 4 års åldern. Orsaken till IH är inte känd men enligt en teori har IH dess ursprung i endotelceller från placentan som implanterar sig hos det nyfödda barnet, i likhet med en benign ”tumörmetastas”.

Frågeställning: Syftet med avhandling var att försöka klargöra uppkomstmekanismer för CA, mer specifikt att försöka besvara frågan huruvida CA är tumörliknande förändringar relaterade till syrebrist i placenta eller äkta tumörer. I samband med det ville vi belysa om det skulle kunna finnas genetiska faktorer som bidrar till genes av CA. Vi ville också undersöka eventuella skillnader mellan små och stora CA och utforska den kliniska betydelsen av de mindre CA. Vidare, vi ville utreda eventuella samband mellan CA och IH.

Material och Metodik: Vårt studiematerial utgjordes av 170 fall av CA (121 från singelbörd och 49 från flerbörd) som identifierades genom särskild kodning i Avd. för Patologins, Karolinska Huddinge, databas 1996–2012. Under denna period undersöktes totalt 15742 placentor enligt consensus definierade kliniska indikationer. Kontrollplacentor (340) rekryterades slumpmässigt från en kohort av 3000 placentor från 2012–2013 och var matchade för gestationslängd och graviditets utgång. Samtliga fall och kontroller genomgick noggrann histopatologisk analys av två perinatalpatologer. Makroskopiska data hämtades från ursprungsrapporter. I analysen undersöktes storlek, lokalisation och histologisk typ av CA samt övriga patologiska drag i placenta, såsom storlek, villusmognad och förekomst av infarkter, intervillösa tromber, avlossning, fetala tromber, tecken till akut eller kronisk inflammation samt patologi av navelsträngen. Maternella basdata och information om graviditet och neonatal utgång extraherades från elektroniska databaser (Obstetrix, Take Care). Genetisk analys genomfördes i en subgrupp av 8 stora CA där färskfrusen CA-vävnad var tillvaratagen. Vi använde s.k. array genomic hybridisation för att jämföra DNA från CA med DNA från frisk placentavävnad från samma patient samt med sammanslaget genomiskt DNA från c: a 100 friska individer (inte placenta). Metoden detekterar strukturella

kromosomavvikelser > 30 kb. För att undersökta samband mellan CA och IH använde vi oss av ett informativt frågeformulär som inkluderade illustrativa foton av IH. Frågeformuläret skickades till 469 mödrar, 153 från fall med CA och 316 kontroller. Statistisk analys genomfördes i studier I, II och IV med olika lämpliga metoder inklusive uni- och multivariat logistisk regressionsanalys. Etiktillstånd har erhållits för samtliga studier.

Resultat/slutsatser:

Studie I: Frekvensen av CA i vår kohort var signifikant högre i flerbörd- jämfört med enkelbördgrupp (2.32 respektive 0.89%). Enkelbördplacentor med CA visade ökad frekvens av hypoxi-relaterade förändringar, såsom acceleration i villusutmognad, infarkter, arteriopathi i decidua, fetal trombos och hypercoiling (tvinning) i navelsträngen. I flerbördplacentor fann vi en signifikant association mellan CA och fetal trombos. För övrigt var histopatologiska drag av CA likartade i enkelbörd- och flerbördplacentor. Frånvaro av samband mellan hypoxitecken och CA i flerbörd kan indikera att dessa placentor härbärgerar ett inbördes hypoxiskt tillstånd som leder till CA uppkomst.

Studie II: Vår analys av 121 enkelbörd- och 49 flerbörd (motsvarande 100 nyfödda barn) placentor med CA visade mycket hög frekvens av preeklampsi i båda grupper. I enkelbördplacentor var förekomst av små CA associerad till maternell preeklampsi och av stora CA till multiparitet och rökning under graviditet. I enkelbördplacentor fann vi ett starkt samband mellan små CA och litenhet vid födseln samt till behov av neonatal intensivvård. Frekvensen av låg APGAR score vid 7 minuter, abnorm pulsatilitetsindex och abnorm blodflödesklass var signifikant högre i enkelbörd jämfört med flerbörd. Våra resultat tyder på att förekomst av CA i placenta kan utgöra en värdefull klinisk-patologisk markör för preeklampsi. Vidare, den gynnsammare neonatalutgång i flerbörd med CA kan avspegla en effektivare adaptiv mekanism mot kronisk syrebrist i dessa placentor.

Genetisk analys av 8 stora CA **(studie III)** visade inga stora strukturella kromosomavvikelser (deletioner, duplikationer) i CA-vävnad med array-metoden. Analysen var däremot inte specifikt riktad mot gener involverade i angiogenes/vaskulogenes och kan inte heller detektera balanserade eller mosaikförändringar. Resultatet talar emot hypotesen att CA är en ”äkta” tumörform, men vidare helgenomstudier måste bekräfta detta och utesluta eventuellt bidrag av mindre strukturella genavvikelser i uppkomst av CA.

Vårt frågeformulär i **studie IV** besvarades va totalt 310 mödrar med svarsfrekvens 66.1%. Vi kunde inte finna någon skillnad i frekvens av IH mellan fall och kontroller eller mellan enkel- och flerbörd. Frekvensen av IH i vårt material var relativt hög (kring 18%), vilket kan förmodligen förklaras av att förekomst av IH var självrapporterad från föräldrar och inte medicinskt verifierad. Vidare, vi kunde inte finna några signifikanta skillnader i frekvens av IH hos nyfödda från graviditet med eller utan preeklampsi och inte heller mellan enkel- och flerbörd. Våra resultat ifrågasätter den tidigare framlagda hypotesen om ett enkelt patogenetiskt samband mellan CA och IH.

9 ACKNOWLEDGEMENTS

I would like to express my gratitude to all who had encouraged and supported me during these years, especially:

My supervisor and good colleague, **Nikos Papadogiannakis,** for believing in me.

Magnus Westgren, my co-supervisor, for your support and calm positive attitude in every situation.

Co-author, **Carl Fredrik Wahlgren,** for great teamwork and expert contribution.

For collaboration **Erik Iwarsson, Anna Lindstrand** and **Maria Pettersson.**

Annette, Annika, Mats and **Gunilla,** from" team perinatal", for support, understanding and flexible work arrangements.

From **the Department of Pathology in Karolinska University Hospital, Huddinge –**

Mikael Björnstedt and **Attila Szakos** for providing me with time and resources to complete my thesis.

Agneta Söderstedt for your diplomatic way of helping me with delicate, sensitive issues and your support.

Märit and **Maazash** for your invaluable help with the archive without any complain.

Peter Lindgren and **Peter Conner** for your assistance with ultrasound images.

Anne Keränen, Sam Ghazi and **Britta Krynitz** for inspiration, valuable advice and help with photos.

Liis Salumäe, my good friend and colleague, for your help, support and for being a great listener.

In memorial, my father, **Vladimir Sirotkin**, the best pathologist in my eyes, for everything. My mother, **Sirje Sirotkina**, for your precious wise words always when I needed them most.

Reeli Sirotkina for being my big sister.

Kostas, my companion for life, for your support, helpful comments and that you cared.

Ioannis for giving true meaning to my life.

10 REFERENCES

1 Fox H, Sebire NJ. Pathology of the Placenta, third ed., Saunders Elsevier. 2007.

2 Amer HZ, Heller DS. Chorangioma and related vascular lesions of the placenta--a review. Fetal Pediatr Pathol. 29(2010)199-206.

3 Ogino S, Redline RW. Villous capillary lesions of the placenta: distinctions between chorangioma, chorangiomatosis, and chorangiosis. Hum Pathol. 31(2000)945–54.

4 Greenberger S, Bischoff J. Pathogenesis of infantile haemangioma. Br J Dermatol. 1(2013)12-9.

5 Mayhew TM. Angiogenesis and villous development in human placenta. J Anat 200(2002)530-531.

6 Nejabati HR, Latifi Z, Ghasemnejad T, Fattahi A, Nouri M. Placental growth factor (PlGF) as an angiogenic/inflammatory switcher: lesson from early pregnancy losses. Gynecol Endocrinol. 27(2017)1-7.

7 Patel J, Landers K, Mortimer RH, Richard K. Regulation of hypoxia inducible factors (HIF) in hypoxia and normoxia during placental development. Placenta. 11(2010)951-7.

8 Veschini L, Belloni D, Foglieni C, Cangi MG, Ferrarini M, Caligaris-Cappio F, Ferrero E. Hypoxia-inducible transcription factor-1 alpha determines sensitivity of endothelial cells to the proteosome inhibitor bortezomib. Blood. 109(2007)2565-70.

9 Guschmann M, Henrich W, Dudenhausen JW. Chorioangiomas-new insights into a well-known problem. II. An immuno-histochemical investigation of 136 cases. J Perinat Med. 31(2003)170-5.

10 Sepulveda W, Wong AE, Herrera L, Dezerega V, Devoto JC. Endoscopic laser coagulation of feeding vessels in large placental chorioangiomas: report of three cases and review of invasive treatment options. Prenat Diagn. 29(2009)201-6.

11 Fan M, Skupski DW. Placental chorioangioma: literature review. J Perinat Med. 42(2014)273-9.

12 Guschmann M, Henrich W, Entezami M, Dudenhausen JW. Chorioangioma--new insights into a well-known problem. I. Results of a clinical and morphological study of 136 cases. J Perinat Med. 31(2003)163-9.

13 Wou K, Chen MF, Mallozzi A, Brown RN, Shrim A. Pregnancy outcomes and ultrasonographic diagnosis in patients with histologically-proven placental chorioangioma. Placenta. 32(2011)671-4.

14 Hoeger PH, Maerker JM, Kienast AK, Syed SB, Harper JI. Neonatal haemangiomatosis associated with placental chorioangiomas: report of three cases and review of the literature. Clin Exp Dermatol. 34(2009)78– 80.

15 Liu H, Gu W, Li X. Natural history and pregnancy outcome in patients with placental chorioangioma. J. Clin. Ultrasound. 42(2014)74–80.

16 Jhun KM, Nassar P, Chen TS, Sardesai S, Chmait RH. Giant chorioangioma treated in utero via laser of feeding vessels with subsequent development of multifocal infantile hemangiomas. Fetal Pediatr Pathol. 34(2015)1-8.

17 Padys P, Fouque L, Le Duff M, D'Hervé D, Poulain P. Propranolol during pregnancy for large chorioangioma. Med Hypotheses. 85(2015)513-4.
18 Jones K, Tierney K, Grubbs BH, Pruetz JD, Detterich J, Chmait RH. Fetoscopic laser photocoagulation of feeding vessels to a large placental chorioangioma following fetal deterioration after amnioreduction. Fetal Diagn Ther.31(2012)191-5.
19 Zanardini C, Papageorghiou A, Bhide A. Giant placental chorangioma: natural history and pregnancy outcome. Ultrasound Obstet Gynecol. 35(2010)332–36
20 Barros A, Freitas AC, Cabral AJ, Camacho MC, Costa E, Leitao H, et al. Giant placental chorioangioma: a rare cause of fetal hydrops. BMJ Case Reports.2 (2011)3880.
21 Rosefort A, Cordier AG, Kaddioui S, Beaumont B, Baergen R, Benachi A, Martinovic J. Co-occurrence of multifocal chorioangiomatosis and mesenchymal dysplasia in preeclampsia. Pediatr Dev Pathol. 16(2013)206-9.
22 Sepulveda W, Alcalde JL, Schnapp C, Bravo M. Perinatal outcome after prenatal diagnosis of placental chorioangioma. Obstet Gynecol. 102(2003)1028-33.
23 Kataria N, Singh A, Bedi PK. Giant Placental Chorangioma: A Rare Case Report. J Clin Diagn Res. 4(2016)ED03-4.
24 Abiramalatha T, Sherba B, Joseph R, Thomas N.Unusual complications of placental chorioangioma: consumption coagulopathy and hypertension in a preterm newborn. BMJ Case Rep. 6(2016). pii: bcr2016215734
25 Vellone VG, Calamaro P, Vignale C, Novaro G, Penna L, Fulcheri E. Atypical Cellular Chorangioma: A Potential Diagnostic Pitfall with Worrisome Aspects but a Favorable Prognosis. Int J Surg Pathol. 23(2015)364-8.
26 Taori K, Patil P, Attarde V, Singh A, Rangankar V. Chorioangioma of placenta: Sonographic features. J. Clin. Ultrasound. 36(2008)113–115.
27 Caldas RT, Peixoto AB, Paschoini MC, Adad SJ, Souza ML, Araujo Júnior E. Giant placental chorioangioma with favorable outcome: a case report and literature review of literature. Ceska Gynekol. 80(2015)140-3.
28 Lim FY, Coleman A, Polzin W, Jaekle R, Habli M, Van Hook J et al. Giant chorioangiomas: perinatal outcomes and techniques in fetoscopic devascularization. Fetal Diagn Ther.37(2015)18-23.
29 Al Wattar BH, Hillman SC, Marton T, Foster K, Kilby MD. Placenta chorioangioma: a rare case and systematic review of literature. J Matern Fetal Neonatal Med. 27(2014)1055-63.
30 Kodandapani S, Shreshta A, Ramkumar V, Rao L. Chorioangioma of Placenta: A Rare Placental Cause for Adverse Fetal Outcome. Case Reports in Obstetrics and Gynecology. (2012)913878.
31 Kesrouani AK, Safi J, El Hajj MA. Rapid Evolution of Placental Chorioangioma: Natural Progression and Outcome. JUM. 3(2013)545–48.
32 Wu Z, Hu W. Clinical analysis of 26 patients with histologically proven placental chorioangiomas. Eur J Obstet Gynecol Reprod Biol. 199(2016)156-63.

33 Reshetnikova OS, Burton GJ, Milovanov AP. Effects of hypobaric hypoxia on the fetoplacental unit: the morphometric diffusion capacity of the villous membrane at high altitude. Am J Obstet Gynecol. 171(1994)1560–1565.
34 Rumboldt T, Eberts PT. Disorders of fetal vascular development: chorangioma, localized chorangiomatosis, chorangiosis, and diffuse multifocal chorangiomatosis. Pathology case reviews. 13(2008)236-240
35 Soma H, Hata T, Oguro T, Fujita K, Kudo M, Vaidya U. Characteristics of histopathological and ultrastructural features of placental villi in pregnant Nepalese women. Med Mol Morphol. 38(2005)92-103.
36 Benirschke K. Recent trends in chorangiomas, especially those of multiple and recurrent chorangiomas. Pediatr Dev Pathol. 2(1999)264-9.
37 Lež C, Fures R, Hrgovic Z, Belina S, Fajdic J, Münstedt K. Chorangioma placentae. Rare Tumors. 2(2010)67.
38 Srinivasan AP, Omprakash BO, Lavanya K, Subbulakshmi Murugesan P, Kandaswamy S. A prospective study of villous capillary lesions in complicated pregnancies. J Pregnancy. (2014)193925.
39 Lifschitz-Mercer B, Fogel M, Kushnir I, Czernobilsky B. Chorangioma. A cytoskeletal profile. Int J Gynecol Pathol. 4(1989)349-56.
40 Miliaras D, Conroy J, Pervana S, Meditskou S, McQuaid D, Nowak N. Karyotypic changes detected by comparative genomic hybridization in stillborn infant with chorionangioma and liver hemangioma. Birth Defects Res (part A). 79(2007)236-41.
41 Benirschke K, Burton GJ, Baergen RN. Pathology of the Human Placenta, sixth ed., Springer 2012.
42 Wurster DH, Hoefnagel D, Benirschke K, Allen FH Jr. Placental chorangiomata and mental deficiency in a child with 2/15 translocation: 46,XX,t(2q-;15q+).Cytogenetics. 8 (1969)389-99.
43 Kim CK, Benirschke K, Connolly KS. Chorangioma of the placenta: chromosomal and electron microscopic studies. Obstet Gynecol. 37(1971)372-6.
44 Fan M, Mootabar H. A rare giant placental chorioangioma with favorable outcome: A case report and review of the literature. J Clin Ultrasound. 18(2014).
45 D'Antonio F, Bhide A. Ultrasound in placental disorders. Best Pract Res Clin Obstet Gynaecol. 28(2014)429-42.
46 Sepulveda W, Aviles G, Carstens E, Corral E, Perez N. Prenatal diagnosis of solid placental masses: the value of color flow imaging. Ultrasound Obstet Gynecol. 6 (2000)554-8.
47 Cvetanovska E, Palmgren Colov N, Kahn R. Chorioangioma of the placenta associated with benign multiple neonatal hemangiomatosis.Acta Obstet Gynecol Scand. 2(2006)243-5.
48 Hosseinzadeh P, Shamshirsaz AA, Javadian P, Espinoza J, Gandhi M, Ruano R, et al. Prenatal Therapy of Large Placental Chorioangiomas: Case Report and Review of the Literature. AJP Rep. 5(2015)e196-202.

49 Cheng YK, Yu SC, So PL, Leung TY. Ultrasound-Guided Percutaneous Embolisation of Placental Chorioangioma Using Cyanoacrylate. Fetal Diagn Ther. 41(2017)76-79.
50 Quarello E, Bernard JP, Leroy B, Ville Y. Prenatal laser treatment of a placental chorioangioma. Ultrasound Obstet Gynecol. 25(2005)299-301.
51 Frieden IJ, Drolet BA. Propranolol for infantile hemangiomas: promise, peril, pathogenesis. Pediatr Dermatol. 26(2009)642-4.
52 Boye E, Jinnin M, Olsen BR. Infantile Hemangioma: Challenges, New Insights, and Therapeutic Promise. J Craniofac Surg. 20(2009)678 – 84.
53 Darrow DH, Greene AK, Mancini AJ, Nopper AJ, Section on dermatology, Section on otolaryngology–head and neck surgery, and Section on plastic surgery. Diagnosis and Management of Infantile Hemangioma. Pediatrics. 136(2015)e1060-e1104.
54 Barnés CM, Christison-Lagay EA, Folkman J. The placental theory and the origin of infantile hemangioma. Lymphatic Research and Biology. 5(2008)245-256.
55 Lo K, Mihm M, Fay A. Current theories on the pathogenesis of infantile hemangioma. Semin Ophthalmol. 24(2009)172-7.
56 Munden A, Butschek R, Tom WL, Marshall JS, Poeltler DM, Krohne SE, et al. Prospective study of infantile haemangiomas: incidence, clinical characteristics and association with placental anomalies. Br J Dermatol. 170(2014)907-13.
57 De Jong S, Itinteang T, Withers AH, Davis PF, Tan ST. Does hypoxia play a role in infantile hemangioma? Arch Dermatol Res. 308(2016)219-27.
58 Ritter MR, Butschek RA, Friedlander M, Friedlander SF. Pathogenesis of infantile haemangioma: new molecular and cellular insights. Expert Rev Mol Med. 29(2007)1-19.
59 Léauté-Labrèze C, Harper JI, Hoeger PH. Infantile haemangioma. Lancet. 12(2017). pii: S0140-6736(16)00645-0.
60 Bruckner AL, Frieden IJ. Infantile hemangiomas. J Am Acad Dermatol. 55(2006)671-82
61 Hemangioma Investigator Group, Haggstrom AN, Drolet BA, Baselga E, Chamlin SL, Garzon MC, Horii KA, et al. Prospective study of infantile hemangiomas: demographic, prenatal, and perinatal characteristics. J Pediatr. 150(2007)291-4.
62 Uebelhoer M, Boon LM, Vikkula M. Vascular anomalies: from genetics toward models for therapeutic trials. Cold Spring Harb Perspect Med. (2012)1;2(8).
63 Chen XD, Ma G, Chen H, Ye XX, Jin YB, Lin XX. Maternal and perinatal risk factors for infantile hemangioma: a case-control study. Pediatr Dermatol. 30(2013)457-61.
64 Jinnin M, Ishihara T, Boye E, Olsen BR. Recent progress in studies of infantile hemangioma. J Dermatol. 37(2010)939-55.
65 Hoornweg MJ, Smeulders MJ, Ubbink DT, van der Horst CM. The prevalence and risk factors of infantile haemangiomas: a case–control study in the Dutch population. Paediatr Perinat Epidemiol. 26(2012)156-62.
66 Kim EJ, Park HS, Yoon HS, Cho S. Maternal and Perinatal Factors of Importance for Occurrence and Severity of Infantile Haemangioma. Acta Derm Venereol. 95(2015)696-9.
67 Tannous Z, Rubeiz N, Kibbi AG. Vascular anomalies: portwine stains and hemangiomas. J Cutan Pathol. 37(2010)Suppl 1:88-95.

68 Boye E, Olsen BR. Signaling mechanisms in infantile hemangioma. Curr Opin Hematol. 16(2009)202-8.
69 John M. Eisenberg Center for Clinical Decisions and Communications Science. Management of Infantile Hemangioma. Comparative Effectiveness Review Summary Guides for Policymakers [Internet]. 21(2016). Rockville (MD): Agency for Healthcare Research and Quality (US); 2011-. Available from: https://www-ncbi-nlm-nih-gov.proxy.kib.ki.se/books/NBK379845/
70 Haggstrom AN, Drolet BA, Baselga E, Chamlin SL, Garzon MC, Horii KA, et al. Prospective study of infantile hemangiomas: clinical characteristics predicting complications and treatment. Pediatrics. 118(2006)882-887.
71 Itinteang T, Withers AH, Davis PF, Tan ST. Biology of infantile hemangioma. Front Surg. 25(2014)1:38.
72 Castaneda S, Garcia E, De la Cruz H, Ramirez O, Melendez S, Sanchez-Palacio J. Therapeutic Effect of Propranolol in Mexican Patients with Infantile Hemangioma. Drugs Real World Outcomes. 3(2016)25-31.
73 Léaute-Labrèze C, Boccara O, Degrugillier-Chopinet C, Mazereeuw-Hautier J, Prey S, Lebbé G, et al. Safety of Oral Propranolol for the Treatment of Infantile Hemangioma: A Systematic Review. Pediatrics. 138(2016).
74 Kaneko T, Sasaki S, Baba N, Koh K, Matsui K, Ohjimi H, et al. Efficacy and safety of oral propranolol for infantile hemangioma in Japan. Pediatr Int. 28(2017).
75 López Gutiérrez JC, Avila LF, Sosa G, Patron M. Placental anomalies in children with infantile hemangioma. Pediatr Dermatol. 24(2007)353–355.
76 Colonna V, Resta L, Napoli A, Bonifazi E. Placental hypoxia and neonatal haemangioma: clinical and histological observations. Br J Dermatol. 162(2010)208-9.
77 Drolet BA, Frieden IJ. Characteristics of infantile hemangiomas as clues to pathogenesis. Does hypoxia connect the dots? Arch Dermatol. 146(2010)1295–9.
78 Mulliken JB, Bischoff J, Kozakewich HP. Multifocal rapidly involuting congenital hemangioma: A link to chorangioma. Am J Med Genet A., 143A(2007)3038–46.
79 Barnés CM, Huang S, Kaipainen A, Sanoudou D, Chen EJ, Eichler GS, et al. Evidence by molecular profiling for a placental origin of infantile hemangioma. Proc Natl Acad Sci USA. 102(2005)19097–102.
80 Itinteang T, Tan ST, Guthrie S, Tan CE, Mcintyre BC, Brasch HD, Day DJ. A placental chorionic villous mesenchymal core cellular origin for infantile haemangioma. J Clin Pathol. 64(2011)870-4.
81 Pittman KM, Losken HW, Kleinman ME, Marcus JR, Blei F, Gurtner GC, Marchuk DA. No evidence for maternal-fetal microchimerism in infantile hemangioma: a molecular genetic investigation. J Invest Dermatol. 126(2006)2533-8.
82 Hoeger PH. Infantile haemangioma: new aspects on the pathogenesis of the most common skin tumour in children. Br J Dermatol. 164(2011)234-5.
83 Mihm MC Jr, Nelson JS. Hypothesis: the metastatic niche theory can elucidate infantile hemangioma development. J Cutan Pathol. 37(2010)Suppl 1: 83-7.

84 Selmin A, Foltran F, Chiarelli S, Ciullo R, Gregory D. An epidemiological study investigating the relationship between chorangioma and infantile hemangioma. Pathol Res Pract. 210(2014)548-53.
85 Bdolah Y, Lam C, Rajakumar A, Shivalingappa V, Mutter W, Sachs BP, et al. Twin pregnancy and the risk of preeclampsia: bigger placenta or relative ischemia? Am J Obstet Gynecol. 198(2008)428.e1-6.
86 Dröge L, Herraìz I, Zeisler H, Schlembach D, Stepan H, Küssel L, et al. Maternal serum sFlt-1/PlGF ratio in twin pregnancies with and without pre-eclampsia in comparison with singleton pregnancies. Ultrasound Obstet Gynecol. 45(2015)286-93.
87 Sibai BM, Hauth J, Caritis S, Lindheimer MD, MacPherson C, M. Klebanoff, et al. Hypertensive disorders in twin versus singleton gestations. Am J Obstet Gynecol. 182(2000)938–42.
88 Krotz S, Fajardo J, Ghandi S, Patel A, Keith LG. Hypertensive disease in twin pregnancies: a review. Twin Res. 5(2002) 8–14.
89 Young BC, Wylie BJ. Effects of Twin Gestation on Maternal Morbidity, Semin Perinatol, 36(2012)162-168.
90 Karumanchi SA. Angiogenic Factors in Preeclampsia: From Diagnosis to Therapy. Hypertension. 67(2016)1072-9.
91 Verdonk K, Visser W, Van Den Meiracker AH, Danser AH. The renin-angiotensin-aldosterone system in pre-eclampsia: the delicate balance between good and bad. Clin Sci (Lond). 126(2014)537-44.
92 Wang A, Rana S, Karumanchi SA. Preeclampsia: the role of angiogenic factors in its pathogenesis. Physiology (Bethesda). 24(2009)147-58.
93 Faupel-Badger JM, Mcelrath TF, Lauria M, Houghton LC, Lim KH, Parray S, et al. Maternal Circulating Angiogenic Factors in Twin and Singleton Pregnancies. Am J Obstet Gynecol. 212(2015)636.e1-636.e8.
94 Maynard SE, Moore Simas TA, Solitro MJ, Rajan A, Crawford S, Soderland P, et al. Circulating angiogenic factors in singleton vs multiple-gestation pregnancies. Am J Obstet Gynecol. 198(2008)200.e1-7.
95 Spiel M, Salahuddin S, Pernicone E, Zsengeller Z, Wang A, Modest AM, Karumanchi SA, Hecht JL. Placental soluble fms-like tyrosine kinase expression in small for gestational age infants and risk for adverse outcomes. Placenta.52(2017)10-16.
96 Kusanovic JP, Romero R, Chaiworapongsa T, Erez O, Mittal P, Vaisbuch E, et al. A prospective cohort study of the value of maternal plasma concentrations of angiogenic and anti-angiogenic factors in early pregnancy and midtrimester in the identification of patients destined to develop preeclampsia. J Matern Fetal Neonatal Med. 22(2009)1021-38
97 Trogstad L, Magnus P, Stoltenberg C. Pre-eclampsia: Risk factors and causal models. Best Pract Res Clin Obstet Gynaecol. 25(2011)329-42.
98 Luo ZC, Simonet F, An N, Bao FY, Audibert F, FraserWD. Effect on neonatal outcomes in gestational hypertension in twin compared with singleton pregnancies. Obstet Gynecol. 108(2006)1138-44.

99 Greco MF, Frieden IJ, Drolet BA, Garzon MC, Mancini AJ, Chamlin SL, et al. Infantile Hemangiomas in Twins: A Prospective Cohort Study. Pediatr Dermatol. 33(2016)178-83.
100 Hubinont C, Lewi L, Bernard P, Marbaix E, Debiève F, Jauniaux E. Anomalies of the placenta and umbilical cord in twin gestations. Am J Obstet Gynecol. 4Suppl(2015)S91-S102.
101 Auger N, Fraser WD, Arbour L, Healy-Profitós J, Drolet BA. Preeclampsia and risk of infantile hemangioma. Br J Dermatol. 12(2016).
102 Pinar H, Sung CJ, Oyer CE, Singer DB. Reference values for singleton and twin placental weights. Pediatr Pathol Lab Med. 16(1996)901-7.
103 Pinar H, Stephens M, Singer DB, Boyd TK, Pflueger SM, Gang DL, Roberts DJ, Sung CJ. Triplet placentas: reference values for weights. Pediatr Dev Pathol. 5(2002)495-8.
104 Stuart A, Amer-Wåhlin I, Gudmundsson S, Maršál K, Thuring A, Källen K. Ductus venosus blood flow velocity waveform in diabetic pregnancies. Ultrasound Obstet Gynecol, 36(2010)344–349.
105 Ley D, Laurin J, Maršál K. Abnormal fetal aortic velocity waveform and postnatal growth. Acta Paediatr. 89(2000)1330-5.
106 Laurin J, Marsál K, Persson PH, Lingman G. Ultrasound measurement of fetal blood flow in predicting fetal outcome. Br J Obstet Gynaecol. 10(1987)940-8.
107 Thuring A, Malcus P, Maršál K. Effect of maternal betamethasone on fetal and uteroplacental blood flow velocity waveforms. Ultrasound Obstet Gynecol. 37(2011)668-72.
108 Stanek J. Hypoxic patterns of placental injury: a review. Arch pathol lab med. 137(2013)706-20.
109 Rajakumar A, Whitelock KA, Weissfeld LA, Daftary AR, Markovic N, Conrad KP. Selective overexpression of the hypoxia-inducible transcription factor, HIF-2alpha, in placentas from women with preeclampsia. Biol Reprod. 64(2001)499-506.
110 Machin GA, Ackerman J, Gilbert-Barness E. Abnormal umbilical cord coiling is associated with adverse perinatal outcome. Pediatr Devl Pathol. 3(2000)462-471.
111 Das S, Ankola P, Chiechi M, Sandhu J. Perinatal Cerebral Arterial Infarction Associated with a Placental Chorioangioma. Am J Perinatol. 25(2008)381-3.
112 Ni Y, Cheng W. Clinical characteristics of early-onset pre-eclampsia in singleton versus multiple pregnancies. Int J Gynaecol Obstet. 132(2016)325-8.
113 Blatt J, Powell CM, Burkhart CN, Stavas J, Aylsworth AS. Genetics of hemangiomas, vascular malformations, and primary lymphedema. J Pediatr Hematol Oncol. 36(2014)587-93.