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PLACENTAL PATHOLOGY REGARDING INFLAMMATION AND A NEW CLASSIFICATION OF STILLBIRTH

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Figure 1 and 2 are painted by Ellen Varli and the flower on next side is painted by Fabian Varli, with some assistance by Matilda Varli.

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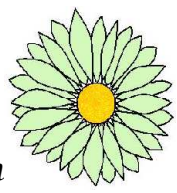
To Morre, Matilda, Ellen and Fabian



ABSTRACT

Background: The incidence of stillbirth has remained almost constant during the last 2-3 decades despite obvious improvements in obstetric care. To achieve a reduction of stillbirth, better understanding of the etiology is imperative. Relevant test protocols and audit work is reported to reduce the number of unexplained cases. Classification of death can help the audit group identifying relevant etiology and causes of fetal death. Since 1954 more than 35 classification systems for perinatal death have been published but there is still no international consensus. Few studies have investigated how causes of stillbirth differ over gestational ages, with varying and conflicting results. Infection is known as an important cause of stillbirth, particularly before gestational week (gw) 28. Various infections are thought to cause different forms of inflammation/infection in placenta. Bacterial infection is supposed to give a maternal inflammatory response in the placenta; histological chorioamnionitis (CAM) followed by a histological fetal response (FIR) including vasculitis (inflammation in vessels in the placenta and/or umbilical cord) and funisitis (inflammation in the Wharton's jelly), whereas a viral infection is supposed to cause chronic villitis. Histological CAM has shown to be clearly associated to stillbirth.

Method: In paper I "the Stockholm classification of stillbirth" is presented and validated regarding inter observer variability. In paper II, a cohort of all 1089 singleton pregnancies in Stockholm during a 12 year period was compared regarding primary and primary + associated cause of death, according to the classification. The cases were divided in early preterm (gw 22+0 to 28+6) and moderately preterm (gw 29+0 to 36+6) plus, term (gw 37+0 to 40+6) and post-term (gw $\geq$ 41+0). Two case-control studies including singleton placentas from 126 term stillborn cases and 273 live born controls (paper III) as well as 112 early preterm (gw 22+0 to 32+6) stillborn cases and 166 gestational week matched references (paper IV) were compared with focus on CAM, FIR and chronic villitis.

Results: We have developed a classification, exclusive for stillbirth, consisting of 17 groups of causes of death and allowing for one primary and several associated causes if needed. Most causes are graded in probability levels (definite, probable, or possible). The validated overall agreement regarding primary cause of death was substantial. When using the classification in the 12 year cohort of stillbirth, almost 90% of cases were ascribed a cause of death. Placental abruption and preeclampsia/hypertension were both more common as cause of death among preterm stillbirths compared to the term/post-term stillbirths, who showed a higher proportion of umbilical cord complications and infection. Infection was more common in post-terms compared to term stillbirths, and in extremely preterm compared to moderately premature stillbirths. In paper III we found CAM (especially severe), chronic villitis, villous immaturity, fetus who was small for gestational age (SGA) and maternal overweight, but not vasculitis or funisitis, to be independently associated with an elevated risk for stillbirth at term. In paper IV we found SGA and "CAM without FIR", but not "CAM with FIR," to be independently associated with a higher risk for stillbirth at early preterm gestation.

Conclusion: The Stockholm classification of stillbirth, primarily meant to be used by audit groups, has showed a low percentage of unclassifiable cases and a low inter observer variability. Knowledge about how causes of stillbirth are distributed over gestational ages could be clinically important and useful in developing strategies for prevention. Results in the case-control studies indicate that the presence of CAM, especially severe, is a risk factor for fetal death in term pregnancies whereas FIR is not. In early preterm pregnancies the presence of CAM is not a risk factor for fetal death if FIR exists, but it is a threat to the fetus if FIR does not occur. Further research is needed to clarify if the development of FIR is actually protecting the fetus from death, a finding that of course could be of great importance for the understanding of the mechanisms of stillbirth.

LIST OF PUBLICATIONS

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Causes of stillbirth at different gestational ages
In manuscript

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Infect Dis Obstet Gynecol. 2012;2012:293867. Epub 2012 Aug 26

Hulthén Varli I, Kublickas M, Papadogiannakis N, Petersson K.
Chorioamnionitis without foetal inflammatory response is associated with stillbirth in early preterm pregnancies
J Matern Fetal Neonatal Med. 2013 Feb 14.
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CONTENTS

LIST OF ABBREVIATIONS AND DEFINITIONS

AOR	Adjusted odds ratio
BMI	Body mass index
CAM	Chorioamnionitis
CI	Confidence interval
CMV	Cytomegalovirus
CODAC	Cause of death and associated conditions
FIR	Fetal inflammatory response
FIRS	Fetal inflammatory response syndrome
Hb	Hemoglobin concentration
ICD	International classification of diseases
ICP	Intrahepatic cholestasis of pregnancy
INCOD	Initial causes of fetal death classification
IUGR	Intrauterine growth restriction
NICE	Neonatal and intrauterine death classification according to etiology
OR	Odds ratio
PSANZ-PDC	Perinatal society of Australia and New Zeeland perinatal death classification
PCR	Polymerase chain reaction
PROM	Premature rupture of membrane
P-PROM	Preterm premature rupture of membrane
ReCoDe	Relevant conditions at death
SGA	Small for gestational age
WHO	World health organization
VUE	Villitis of unknown etiology

Antepartal	Before labor starts
Early fetal death	Fetal death at gestational week 22+0 to gestational week 27+6
Early neonatal death	Death before seven days of life among liveborn infants
Extremely preterm birth	Birth before 28 completed weeks of gestation
Infant mortality	Death of an infant less than one year of age
Intrapartal	During labor
Late fetal death	Fetal death at 28 completed weeks of gestation or later
Neonatal death	Death before 28 days of life among liveborn infants
Perinatal mortality	Stillbirth and early neonatal death
Preterm birth	Infant born before 37 completed weeks of gestation
Small for gestational age	Birth weight <2 standard deviation below the mean birth weight
Stillbirth	Fetal death at 22 completed weeks of gestation or later (before 1 of July 2008 the Swedish definition was fetal death at 28 completed weeks of gestation or later)

1 BACKGROUND

1.1 STILLBIRTH

1.1.1 Definitions, Trends and Incidence

The incidence of perinatal mortality (stillbirth and early neonatal death) has declined in Sweden over the last decades. In 1973 the incidence was about 14 per 1000 births and until 1985 it declined to approximately 7 per 1000 births, of which 3-4 were stillbirths[1]. This effect is suggested to depend on improvements in obstetric and neonatal care, such as antenatal corticosteroids for lung maturation, surfactant therapy, assisted ventilation and the use of biochemical and electronic fetal monitoring during delivery. Even if important improvements have been made in obstetric care, the results are mostly seen in the neonates with, in particular, a decreased incidence in early neonatal death (death in the first week of life), as well as a decrease in intrapartal death (death during delivery), whereas antepartal death (death before start of labor) has been more stable. The incidence of the total neonatal death (death before 28 days of life) has continued to decline and in 2009 the incidence was 1.6 per 1000 live births [2]. In contrast the incidence of stillbirth has remained almost constant during the last 2-3 decades despite obvious improvements in obstetric care. The reasons for this are not clear and might depend on several, potentially counteracting factors; one explanation, for example, might be that better obstetric care leads to more women with severe illness, previously advised not to get pregnant, managing to advance through pregnancy today; another possibility is the change in the demographics of pregnant women, with an increase in maternal age and maternal body mass index (BMI), both known risk factors for stillbirth [3].

In 2009 the incidence of stillbirth and late fetal death (from gestational week 28) was 2.9 per 1000 births [2]. This is a low figure compared to the rest of the world, where the rate of late fetal death varies greatly from 2.0 per 1000 total birth in Finland to more than 40 per 1000 total births in Nigeria and Pakistan [4].

Until June 2008 stillbirth in Sweden was defined as fetal death from gestational week 28+0. The definition was then changed to include fetal deaths from gestational week 22+0, to concord with international praxis. This definition is in line with the recommendations of the International Classification of Diseases (ICD-10) [5] and WHO [6] for national perinatal statistics: to include all fetuses and infants weighing 500 g or more at birth or, if birthweight is unknown, fetuses with gestational week 22+0 or more; or, if both these criteria are unknown, fetuses with a body length of 25 cm or more [6, 7]. To facilitate international comparison WHO recommends countries to report late fetal deaths, defined as a birthweight of 1000 g or more or, if birthweight is unknown, 28 completed weeks of gestation or, if both factors are unknown, a body length of 35 cm or more. The reason for recommending countries to also include early fetal deaths into national statistics is to increase the frequency of reported late fetal death [4].

The incidence of stillbirth from ≥ 22+0 week of gestation in Sweden, in 2009, was 4.1 per 1000 births [2], which is low compared to many other countries; especially considering that in most other countries a birth weight ≥ 500g is used as a definition, resulting in approximately 15% lower incidence [8]. Of the total perinatal deaths in Sweden, more than 70% consist of stillbirths [2].

Worldwide there are at least 2.65 million late fetal deaths every year (98% occurring in low- and middle- income countries), a number only slightly lower than the yearly 3 million early neonatal deaths and actually larger than the yearly number of all deaths caused by HIV/AIDS [4, 9]. This makes stillbirth an important health problem that needs global attention and increased resources to achieve substantial improvement in health care systems, infrastructure and education, and research.

In all the papers included in this thesis we have used gestational age ≥ 22+0 as the definition of fetal death/stillbirth. In the majority of cases the gestational age was determined by routine ultrasound at gestational week 18-20, but if no ultrasound was performed it was calculated from the first day of the last menstrual period.

1.1.2 Classification of stillbirth

In order to achieve a reduction of stillbirth better understanding of the etiology is imperative [10-12]. In the literature, the proportion of stillbirths without a known cause of death varies from 7 to 82% [10, 11, 13-24]. Many groups have reported that audit work as well as relevant test protocols reduce the number of unexplained cases [13, 15, 20, 25, 26]. Audit groups are important in the learning process, as they help us develop and implement guidelines in healthcare, identifying avoidable factors and optimizing medical care. To identify relevant etiology and causes of fetal death the audit groups need a well-developed classification that helps understanding and elucidating complex aspects of stillbirth, providing suggestions for planning of new pregnancies and developing new preventive and treatment strategies.

Since 1954 more than 35 classification systems for perinatal death have been published [10-12, 16-19, 27-56] and there is no international consensus on which one to use. The user friendliness for each of the classification varies and they focus on different aspects, as immediate cause of death [18], underlying cause of death [16, 17, 27, 31, 34, 35, 38-44, 54, 57-59], pathological findings [29, 30, 32, 44], epidemiological concerns [10, 12, 32, 35, 36, 39, 45, 53] and if the death was avoidable or unavoidable [10, 31, 33, 38, 39, 45, 46, 57].
Most classification systems are developed for perinatal death. Inclusion of stillbirths and neonatal deaths in the same classification can be inadequate and confusing, since they may have different causes of and associations to death, even though there is an obvious biological overlap.

Different quality aspects must be taken into account when designing a classification system. De Galan-Roosen [17] as well as many other authors [10, 12, 29, 31] have raised concerns about this. The most important suggestions are that the classification should be easy to use for clinicians, have a low inter-observer variability, be easy to expand

in terms of sub-classification, be based on clinical factors and autopsy findings including histology of the placenta, explain the underlying cause of death (the factor that initiated the chain of events leading to death), illustrate associated factors which may have contributed to the death, result in a high percentage of classifiable cases and identify possibilities of reducing the perinatal mortality. Moreover, it is important to have strictly defined criteria for each group in order to be able to compare different populations.

The oldest classifications of perinatal death were clinical, ascribing the death to maternal diseases or obstetric complications [60, 61]. When autopsies came to be performed systems based on autopsy findings superseded those based on presumed etiology [62-64]. Since the fifties, there have been two parallel approaches in classifications, one built on obstetric antecedents or clinico-pathological background and the other built on fetal/neonatal factors.

1.1.2.1 Classifications by obstetric factors

In Scotland, in 1954, Baird [27] developed the "**The Aberdeen classification**" (Table 1), built on clinical conditions instead of autopsy since the authors thought it was important to identify the factor that initiated the chain of events leading to death instead of what killed the infant in the end. Baird and colleges claimed that autopsy usually only indicates *how*, not *why*, the infant dies, and at that time autopsy failed to explain the cause of death in 40% of cases [27]. The authors used the classification to review 1008 singleton perinatal deaths in Aberdeen Maternity hospital in 1938-1952 [27] with the following distribution of causes: Premature (birthweight < 2.500 g) with unknown cause 20%, Mature with unknown cause 14%, Trauma 19%, Toxemia 10%, Ante-partum hemorrhage 11%, Maternal disease 6%, Fetal deformity 16% and Other causes 5%. In 1969 the classification was renamed to "**The Amended Aberdeen classification**" [65] (Table 1) where the preterm and mature unknown groups were added together as unknown (even if they existed as two subgroups) and it was expanded with three groups: serological incapability, infection of fetus or infant and unclassified (when information was inadequate).

In 1986 **Cole at al** [40] (Table 1), suggested that the unknown group should be divided into "unexplained low birthweight (< 2.5 kg)" and "unexplained normal birthweight (≥ 2.5 kg)" since it had been showed that those two groups of death had different epidemiological characteristics with different implication for prevention. Since infections were only found in a small amount, the group was erased as an own group and instead included in "other causes".
The Amended Aberdeen was later revised by Whitfield et al. 1986,[34] the "**Whitfield classification**" (Table 1), in which the perinatal death group was renamed to "total perinatally related wastage" and extended to also include: late abortions (after 20 weeks gestation except termination due to fetal abnormities), all neonatal deaths and all infant deaths (one month to one year of age).

Table 1. Classifications by obstetric factor.

Aberdeen classification (1954) [27] • Premature, cause unknown • Mature, cause unknown • Trauma • Toxaemia • Ante-partum haemorrhage • Foetal deformity • Maternal disease • Other causes
Amended Aberdeen classification (1969) [65] • Malformation • Serological incompatibility • Mechanical causes • Cause of death uncertain • Toxaemia • Ante-partum haemorrhage (without toxemia) • Maternal disease • Infection of foetus or infant • Miscellaneous defined causes • Unclassified (Information Inadequate)
Cole et al classification (1986) [40] • Congenital anomaly • Isoimmunization • Pre-eclampsia • Antepartum haemorrhage • Mechanical • Maternal disorder • Miscellaneous • Unexplained ≥ 2.5kg • Unexplained < 2.5kg
Whitfield classification (1986) [34] • Spontaneous preterm • Intrauterine growth retardation • Unexplained intrauterine death • Intrapartum asphyxia Trauma • Hypertension • Maternal disease • Antepartum haemorrhage • Fetal abnormality • Hemolytic disease • Infection • Other

1.1.2.2 Classifications by Fetal/Neonatal factor

In 1956 **Bound et al** [29] published a classification (Table 2) developed on autopsy findings, as they claimed this would be less susceptible to differences of opinion than

one based on clinical data alone. **Butler and Bonham** [30] later did some minor modification to the Bound classification (Table 2) for use in the British perinatal mortality survey, where more than 7000 perinatal deaths in 1958 were investigated in Great Britain [30, 66]. In 1980 Wigglesworth, a British perinatal pathologist, suggested that perinatal deaths should be classified in to five simplified pathological groups with a sub-classification by birthweight [12] (Table 2). He meant that classifying only by clinical causes can be inadequate because several of the definitions are highly subjective and do not provide the necessary information relating to management, but he also claimed that classifying by primary autopsy findings is limited because not all cases will be autopsied and when a number of findings are identified by the pathologist it can be hard to get uniformity as to the major cause [12]. The Wigglesworth classification has been used widely and revised in both Great Britain and other countries [16, 35, 36, 51, 56, 67-72] and an "**Extended Wigglesworth classification**" (Table 2) was presented in 1986 [73]. In 1986 Hey et al.[32] further developed the Bound [29]/Butler and Bonham [30] classification and the Wigglesworth classification [12] to "**The Fetal and Neonatal Factors classification**" (Table 2), where they also included a classification flow chart. The authors suggested that further sub-classification, that would probably be necessary, could be achieved in a flexible way by using ICD codes.

Table 2. Fetal/Neonatal Classifications

Bound classification (1956) [29] • Ante-partum death with maceration only • Intra-partum asphyxia • Congenital malformations • Birth trauma • Pulmonary syndrome of the newborn • Ante-partum asphyxia • Pneumonia • Erythroblastosis foetalis • Intraventricular haemorrhage • Miscellaneous • Previability
Butler and Bonham classification (1963) [30] • Congenital malformation • Isoimmunization • Ante-partum death with autopsy evidence of anoxia • Ante-partum death without autopsy evidence of anoxia • Intrapartum death with autopsy evidence of anoxia • Intrapartum death with autopsy evidence of anoxia and trauma • Intrapartum death without autopsy evidence of anoxia and trauma • Cerebral birth trauma • Neonatal death (no autopsy abnormality) • Hyaline membrane • Intraventricular haemorrhage • Pulmonary infection • Extrapulmonary infection • Massive pulmonary haemorrhage • Miscellaneous • No necropsy

Wigglesworth classification (1980) [12] • Normally formed macerated • Congenital malformations • Condition associated with immaturity • Asphyxial condition developing in labour • Specific condition other than above
Fetal and neonatal factors classification (1986) [32] • Congenital anomaly • Isoimmunization • Antepartum asphyxia • Intrapartum asphyxia • Birth trauma • Pulmonary immaturity • Hyaline membrane disease • Intracranial haemorrhage • Infection • Miscellaneous • Unclassified or unknown
Extended Wigglesworth (1986) [73] • Congenital defect/malformation (lethal orsevere) • Unexplained antepartum fetal death • Death from intrapartum ʻasphyxiaʼ, ʻanoxiaʼ or ʻ traumaʼ • Immaturity • Infection • Other specific causes • Accidental or non-intrapartum trauma • Sudden infant death, cause unknown • Unclassified

1.1.2.3 Other classifications

In 1977 the pathologist Naeye [44] made a pathological classification, including twenty causes of death, based on the underling obstetric cause of death. The classification was used in "**the US Collaborative Perinatal Project**" [44] analyzing 1.993 deaths (fetal deaths from gestational week 20 and all neonatal deaths) between 1959 and 1966 in 12 university-affiliated hospitals in different regions of the United States. The most common causes of death were: amniotic fluid infections (17%), placental abruption (11%), premature rupture of membrane (PROM, 9%), congenital anomalies (6%) and large placental infarction (≥ 25%; 6%). In 20% there was no known cause. Chan et al. [31] commented that this classification requires adequate clinical information, autopsy and placental examination in a high percentage of cases, which, today, should be evident to most obstetricians considering perinatal death cases. The absence of clear definitions in the Naeye classification is a disadvantage but for the first time there is a group called "severe fetal undernutrition" in a classification that probably includes "intrauterine growth restriction" (IUGR) fetuses and infants; however since that group only included 0.1% of the perinatal deaths most IUGR fetuses must have been included in other groups, such as "placental markedly growth retarded" (2.3%) and "large placental infarcts" (6.1%). In **1983 Hovatta et al** [19] composed a preliminary classification for stillbirth only. It was based on both autopsy and clinical findings after

analyzing 243 stillborn fetuses (born after 26 gestational weeks) in Helsinki, Finland, between 1974 and 1979. The Hovatta classification contains ten main groups (two with subgroups). Their study had only 9.1% unexplained cases, even if it probably had been more correct to include the "asphyxia for unexplained reason" with 3.3% in the unexplained group, giving in total 12.4% as unexplained; those figures for unexplained cases are amongst the lowest in published literature [19]. The low frequency of unexplained cases was probably due to a careful investigation including autopsy and examination of the placenta together with thorough consideration of each case by both an obstetrician and a perinatal pathologist. Unfortunately, the Hovatta classification, similarly to the Naeye classification, did not include definitions of the causes and there was no cause of death due to IUGR which is nowadays widely recognized as an important contribution or risk factor in stillbirth.

In 1992 Fretts et al.,[11] developed a **Classification of Primary Cause of Fetal Death** with definitions for each cause of death. The purpose of their study was to evaluate changes in cause of death, over three decades (1961-1988) in a tertiary care unit in Montreal, Canada. The primary causes were assigned in audit and the authors recognized that more than one factor might have contributed to the fetal death. Changes concerning cause of death over the decades studied were: Rh isoimmunization and birth asphyxia almost disappeared; unexplained antepartum fetal death and death due to IUGR declined; death due to infection and placental abruption did not change neither did death due to toxemia and diabetes even if those only contributed to a small part of the fetal death. The risk of fetal death was increased in women with hypertension or diabetes in the sixties but in the eighties it was only increased among women with insulin depended diabetes. The classification presented in this thesis is essentially a further development of the Fretts classification.

The **Nordic/Baltic perinatal death classification** by Langhoff-Roos et al.,[33, 52] focuses on potentially avoidable perinatal death and thereby identifies suboptimal care. It is based on five variables: fetal malformation (the only category that has priority over the others); time of death in relation to delivery; birthweight (growth retardation); gestational age; Apgar score ($< / \geq 7$ at 5 minutes). By using those five variables there will be a total of 13 categories where some, according to the authors, represent potentially avoidable deaths (i.e. antenatal death in single IUGR fetus $\geq$ 28weeks; intrapartal death after $\geq$ 28weeks; neonatal death in preterm, 28-33 weeks, with Apgar score ≥ 7 at 5minutes).

Neonatal and Intrauterine Death Classification according to Etiology (NICE), a Swedish computer based hierarchical and etiologically oriented classification system based on a modified Wigglesworth classification, was developed by Winbo et al. 1997 [35, 36]. The authors used four national medical registries (the Medical Birth Registry; The Registry of Congenital Malformation; The Cause of death Registry and The Registry of Congenital Cardiac Defects), linked by the mother's personal identification number and the date of birth. This classification is most useful for large scale epidemiological studies.

1.1.2.4 Classifications in 21st century

Probably the most known and used classifications worldwide are the Amended Aberdeen [65], Wigglesworth [12], and Extended Wigglesworth [73]. However, during the 21 century there are at least five new classifications, aside from the classification presented in this thesis.
In Australia and New Zeeland a national perinatal death classification has been developed, **the Perinatal Society of Australia and New Zeeland Perinatal Death Classification (PSANZ-PDC)** [31] (Table 3). It is based on the obstetric antecedent factors that initiated the chain of event leading to death; it is hierarchical in descending order but strict only regarding congenital anomalies and it includes specific definitions and guidelines.

In Great Britain Gardosi et al.,[18] 2005, published a classification for stillbirth only, **Relevant Conditions at Death (ReCoDe)** (Table 3). It is hierarchical (starting from conditions affecting the fetus and moves outwards in simple anatomical groups: umbilical cord, placenta, amnion fluid, uterus, mother, intrapartum, trauma and unclassified); the classification identifies the relevant condition at the time of death, "what went wrong, not necessarily why" but also allows for one more condition if relevant.

The Dutch **Tulip classification** (Table 3) from 2006 by Korteweg et al.,[10], is a classification for stillbirth, neonatal and perinatally related infant death (during hospital admission from birth onwards). It separates cause and mechanism of perinatal mortality for the purpose of counseling and prevention. It identifies the underlying cause of death, defined as the initial pathophysiological entity initiating the chain of events leading to death and the mechanism of death defined as the organ failure that has directly led to death. It also includes the origin of mechanism that they call "process" i.e.: if a fetus dies due to umbilical cord prolapse this will be the cause and cardio circulatory insufficiency will be the mechanism of death.

Cause of Death and Associated Conditions (CODAC) (Table 3) is a Norwegian classification for perinatal death by Frøen al.,[38] that identifies the underlying cause of death. The classification contains ten main categories and 94 subcategories that are further specified in further 577 categories, which enable categorization of important information to explain perinatal death for the purposes of counseling and prevention through audit, epidemiology, and research. An update of the classification is planned during 2013.

The latest classification system is **the Initial Causes of Fetal Death classification (INCODE)** (Table 3) developed in 2010 by the Stillbirth Research Collaborative Network [39], a consortium of five academic health centers in United States working with stillbirths. The classification has strict definitions and divides causes of death into probable (high likelihood of directly cause of the fetal death) and possible (not a direct cause of the fetal death but possibly involved in a pathophysiological sequence

leading to the fetal death). If a condition does not meet the criteria for probable or possible causes of death but is considered as potentially important it is recorded as present. The classification allows for more than one cause of death without forcing the choice of a single primary cause of death. Causes of death were grouped into six broad categories for purposes of analysis.

Tab 3. Classifications in 21st century

PSANZ-PDC Classification (2004) [31]
1. Congenital abnormality (including terminations for congenital abnormalities) 2. Perinatal infection 3. Hypertension 4. Antepartum hemorrhage (APH) 5. Maternal conditions 6. Specific perinatal conditions 7. Hypoxic peripartum death (typically infants of >24 weeks gestation or >600g birthweight) 8. Fetal Growth Restriction (FGR) 9. Spontaneous preterm (<37 weeks gestation) 10. Unexplained antepartum death 11. No obstetric antecedent
ReCoDe classificatioan (2005) [18]
1. Fetus 2. Umbilical Cord 3. Placenta 4. Amniotic fluid 5. Uterus 6. Mother 7. Trauma **8.** Unclassified
Tulip classification (2006) [10]
1. Congenital anomaly 2. Placenta 3. Prematurity/immaturity 4. Infection 5. Other 6. Unknown
Simplified CODAC (2009) [38]
1. Infection 2. Neonatal 3. Intrapartum 4. Congenital anomaly 5. Fetal 6. Cord 7. Placenta 8. Maternal 9. Unknown 10. Termination
INCODE (2010) [39]
1. Maternal Medical Conditions during Pregnancy 2. Obstetric Complications 3. Maternal or Fetal Hematologic Conditions 4. Fetal Genetic, Structural, and Karyotypic Abnormalities 5. Placental and/or Fetal Infection (Excluding Fetal Membranes) 6. Pathologic Placental Conditions 7. Other Pertinent Condition Not Specified in Sections

Four of the latest classifications (PSANZ-PDC, ReCoDe, Tulip and CODAC) together with the Amended Aberdeen and Extended Wigglesworth were evaluated quite recently [21] considering: retaining relevant information, ease of use, inter-observer reliability and amount of unexplained cases. Overall CODAC preformed best and PSANZ-PDC and ReCoDe performed well. Tulip demonstrated the best agreement and a low proportion of unexplained stillbirths. The researchers could not, according to the study result, recommend the Amended Aberdeen and the Extended Wigglesworth for classification of stillbirth.

1.1.3 Risk factors for fetal death

Obstetricians have been long aware of a variety of risk factors associated with stillbirth. In a recent multivariate analysis of risk factors in high income countries maternal overweight and obesity (BMI, >25 kg/m^2), advanced maternal age (>35 years), maternal smoking, primiparity and small for gestational age (SGA) were shown to be the most important risk factors for stillbirth; however, low education, low socioeconomic status and no or inadequate antenatal care remained important contributors to stillbirths even in high income countries [3]. In Sweden, as in other high income countries, women are delaying childbearing. Mean age of primiparas was approximately 24 years in 1975 and increased to 28 years in 2010, and the proportion of women with BMI $\geq$ 25 at first antenatal visit has increased from 25% in 1992 to almost 37% in 2009 [2]. Primiparity cannot be eliminated as a risk factor but overweight and delaying child birth are both modifiable risk factors. Daily smoking in early pregnancy has fortunately declined in Sweden from 31% in 1983 to about 7% in 2010; it has declined in all age groups but unfortunately it is most common in the youngest group, with 19% of women <19 years state smoking at gestational week 30-32 [2]. It has been shown that women who stop smoking in their first trimester have a comparable risk for stillbirth with women who did not smoke in earlier pregnancies [74]; thus a "stop smoking program" in antenatal care will feasibly result in reduction of stillbirth risk. In Sweden SGA is defined as birth weight < 2 standard deviations below mean[75] but in a lot of other countries as birthweight $\leq$ 10th percentile for their gestational age [76]. SGA is a well-known risk factor for stillbirth [3, 77]. It is important to distinguish SGA from intrauterine growth restriction; although the group of SGA fetuses obviously contains growth restricted infants about 30% of the SGA fetuses are not growth restricted [78]. The SGA group includes fetuses that are associated with, for example, maternal smoking, low educational level, nulliparity, advanced maternal age, maternal disorders (i.e. hypertensive disorders, diabetes and chronic renal diseases), placental dysfunction or fetuses that are genetically/constitutionally small. Many of the above mentioned risk factors for SGA are shared with stillbirth [78]. About half of unexplained stillbirths have a birthweight < 10th percentile (corrected for gestational age and parental characteristics)[79]. To achieve reduction in stillbirth rate it is important to find the true growth restricted fetus, since stillbirth associated with intrauterine growth restriction, without any other obvious direct cause, is one of the major types of stillbirth [18, 77, 79].

Other risk factors for stillbirth not discussed in detail here include: previous stillbirth, multiple gestation, Afro-American ethnicity, poor nutritional status and post-term gestation [80].

1.1.4 Causes of stillbirth

It is not always obvious as to what should be defined as a cause of stillbirth and what should be defined as a risk factor for stillbirth; moreover in many previous publications this distinction is not made and only the cause of death is referred to. Here we have included the most commonly described or accepted causes of (or associations to) stillbirth.

1.1.4.1 Malformation and chromosomal abnormalities

Malformation and chromosomal abnormality are associated with increased risk of perinatal death but the causal relationship between an identified abnormal finding and fetal death is not always obvious [37]. The influence of malformation and chromosomal abnormality as a cause of stillbirth has decreased [11, 81], probably because of early diagnosis and pregnancy termination when lethal anomalies, such as anencephaly and chromosomal disorders are discovered [11, 82]; still, malformations and chromosomal abnormalities contribute to a large part of stillbirth. In Stockholm 1998-99, 10% of stillbirth ≥ 22 gestation weeks were due to malformation or chromosomal abnormality. To identify malformation and chromosomal abnormalities both autopsy and cytogenetic investigation are important. With autopsy Pauli et al.[83] identified almost 25% of stillborn infants (from 20 weeks of gestation) to have congenital anomalies as cause of death. In a study by Korteweg et al. [84], 5% of stillbirth infant without obvious malformation showed chromosomal abnormalities with cytogenetic analyses.

1.1.4.2 Infection

Infections (bacterial, viral and protozoal) are important causes of stillbirth. In developing countries up to 50% of stillbirths are probably caused by infections, compared to 10-25% in high-income countries [85].

The relationship between maternal infection and stillbirth is not always clear [37, 86, 87]. Firstly, finding microorganisms in the placenta or fetus does not prove causality, for example a lot of microorganisms can be contaminants during delivery. Secondly, infection-caused stillbirth may manifest with symptoms that are not readily appreciated or appear to be related to infection; for example parvovirus causing hydrops and toxoplasmosis causing hydrocephalus. Thirdly, some organisms may not be easily identified by commonly used detection methods, for example Ureaplasma, Mycoplasma and certain viruses [88 89, 90]. Lastly, serologic tests even when positive do not always prove causality.

Infection can theoretically cause fetal death by several mechanisms [87, 91]: 1) Serious systemic illness of the mother (high maternal fever, respiratory distress or other systemic reactions) might cause fetal death without the organisms being transmitted

to the placenta or fetus [92, 93]. 2) The placenta can be infected, resulting in reduced blood flow preventing oxygen and nutrients from crossing to the fetus [94, 95]. 3) The fetus can get infected, through placenta or membranes, leading to damage of vital organs, for example lung [96], heart or brain; alternatively, in early pregnancy fetal infection can cause a congenital deformity that can be incompatible with life, for example rubella [97].4) Maternal infection of the genital tract or elsewhere might precipitate preterm labor that the fetus is unable to tolerate [98].

Placental and fetal infections likely originate from two predominant pathways. The most common is an ascending infection from the vagina through the cervix to the fetal membranes, amnion fluid and fetus, leading to acute chorioamnionitis CAM, vasculitis and funisitis; infection may also arise systemically in the mother, spread hematogenous, and reach the fetus through the placental parenchyma (villi) [37, 99].

The most important infection worldwide concerning stillbirth is probably syphilis, caused by the spirochete Treponema pallidum. Occurrence of syphilis in women of reproductive age ranges from 0.02% in high income countries to 12% in some African populations [100]. In untreated mothers with syphilis about 45% of the fetus will die in utero and another 30-40% will be born alive but with congenital syphilis[100]. If antenatal screening for syphilis is performed the incidence of perinatal death attributable to syphilis could be reduced by about 50% [101].

Malaria is another important global cause of stillbirth[100], even if the causal relationship between malaria infection and fetal death has been difficult to demonstrate. Malaria is associated with IUGR, preterm birth, stillbirth, severe maternal anemia and even maternal death, where primiparas with primary infection have the worse outcome[102-104]. The pregnancy outcome is related to the extent of placental malaria that results in placental insufficiency and in part to the degree of maternal anemia. The placenta insufficiency is caused by lymphocyte and macrophage accumulation, resulting in thickening of placental trophoblast membrane impeding blood flow through the placenta and leading to restricted transport of oxygen and nutrients [94]. In a recent review placental malaria was associated with a twofold increase in the risk of stillbirth [105].

Other examples of infectious organisms involved in stillbirth are [100]: Bacterial: Group B streptococcus, E coli, Klebsiella, Enterococcus, Listeria monocytogenes, Ureaplasma urealyticum, Mycoplasma hominus etc; Viral: most commonly parvovirus B19, cytomegalovirus (CMV) and enteroviruses: Protozoa, such as Trypanosoma cruzi and Toxoplasmosis gondii; and, fungi, such as Candida albicans in case reports.

1.1.4.3 Immunization

Erythrocyte immunization of the fetus is caused by incompatibility between maternal and fetal erythrocyte antigens (mainly RhD) leading to destruction of fetal erythrocytes, anemia, hydrops and heart failure. It was earlier a common cause of stillbirth but after introduction of anti-D prophylaxis in the 1960s it is now rare; still, incompatibility for

minor erythrocyte antigens such as Rhc or Kell occasionally occurs and may be cause of immunization and stillbirth [106]. Platelet alloimmunization caused by incompatibility between fetal and maternal platelet antigen is a rare but important cause of intracranial hemorrhage and stillbirth [107, 108].

1.1.4.4 Feto-maternal transfusion

Abdominal trauma and placental abruption are among others risk factors for feto-maternal transfusion but usually no underlying cause is identified [109]. Feto-maternal transfusion can be acute or chronic [110]. Acute feto-maternal hemorrhage leads to severe fetal anemia and hypovolemia that can result in fetal death. Chronic bleeding is associated with chronic hypoxia leading to neurologic impairment or stillbirth [109, 110]. The threshold for feto-maternal hemorrhage which is severe enough to cause stillbirth is unknown but it has been shown that the fetus can tolerate an acute hemorrhage of 40 percent of the blood volume [111].

1.1.4.5 Birth hypoxia

Intrapartal stillbirth is uncommon in Sweden with an estimated incidence of 1.9/ 10.000 birth; in 90% of the cases it is reported to be caused by birth hypoxia [112]. Birth hypoxia is caused by for example shoulder dystocia, umbilical prolapse, placental abruption and abnormal uterine contractions (hyperstimulation) mostly due to overstimulation by oxytocin. The incidence of intrapartal stillbirth in other developed countries is estimated to be around 1/1000 births and in developing countries around 7/1000 births. In some southern African and Asian countries the incidence is even as high as 20-25/1000 births [113].

1.1.4.6 Intrauterine growth restriction (IUGR)/placental insufficiency

IUGR can be defined as the failure of the fetus to reach its genetic growth potential and is one of the most common causes of stillbirth [18]. IUGR is multifactorial and rather a sign of an underlying illness than an individual entity [18, 37, 114]. The most common cause (25-30%) of IUGR is placental insufficiency, caused by abnormal placentation where a deficient trophoblastic invasion occurs [114]. The trophoblastic invasion is needed to transform the spiral arteries into distended flaccid vessels. The failure of trophoblastic invasion leads to spiral arteries retaining their contractility and results in impaired utero-placental blood flow [114].
Beside placental insufficiency IUGR can be caused by: chromosomal alterations, genetic syndromes, intrauterine infections (i.e.: rubella virus, CMV, varicella zoster virus and Toxoplasma gondii), maternal disease (i.e. insulin-dependent diabetes mellitus with vasculopathy, cyanotic cardiopathies, restrictive lung disease, severe renal conditions, autoimmune diseases, hereditary or acquired thrombophilia, hyperhomocysteinemia and severe anemia) or nutritional disorders [114]. An underlying cause of IUGR cannot be identified in a significant proportion of cases (idiopathic IUGR) [18].
IUGR is, besides stillbirth, connected to other forms of adverse perinatal outcome as neonatal death, premature births, asphyxia, meconium aspiration, cognitive

dysfunction and cerebral palsy [114]. In recent years, epidemiological studies have established that suboptimal intrauterine growth environment, as in IUGR, confers a significant risk for development of cardiovascular disease or metabolic syndrome (possibly even other diseases) in adult life [115].

SGA is often used as a proxy for IUGR and most commonly population-based birthweight standards are used to identify SGA fetuses. Later studies have shown customized-based birthweight standards (based on maternal height, weight, parity, ethnic background and fetal gender) having an improved capacity to identify fetuses with increased risk of IUGR associated adverse perinatal outcome [116-118].

1.1.4.7 Umbilical cord complication

Umbilical cord complication, leading to compromised fetal blood flow, is a common perinatal occurrence. It is identified as a cause of stillbirth but possibly underreported due to earlier lack of histological diagnostic criteria [119]. In 2008 Parast et al., proposed histological diagnostic criteria including vascular dilatation and thrombosis (within the umbilical cord, chorionic plate, and/or stem villi) as "minimal criteria" for suggesting the diagnosis of cord accident; for a probable diagnosis they required the previous findings as well as regional distribution of avascular villi or villi showing stromal karyorrhexis [120]. Umbilical cord complication is a heterogeneous group including acute and chronic causes [119]. Acute causes are for example cord prolapse (associated with abnormal presentation, prematurity, multiparity, obstetric manipulation, membrane rupture and abnormally long umbilical cords) [121-123] and rupture of vasa previa, where fetal vessels traverse the fetal membranes over the internal cervical os (as a result of either velamentous cord insertion or presence of accessory lobes [124]. Velamentous cord insertion [124] and other vascular abnormalities of the placenta, such as hypo/hypercoiling of the cord, may be related to suboptimal placentation in early pregnancy and associated to placental pathology causing stillbirth [125-127].

Causes that can be both acute and chronic are true knots, tight cord around body or torsion (probably more frequently occurring if the umbilical cord is uncoiled or abnormally long) [121, 122, 124]. Umbilical cord complications of more chronic nature are for example hyper- or hypocoiling and velamentous (or marginal) insertion. The presumed mechanism for injury secondary to chronic cord complication is impaired circulation, gradually leading to hypoxia and fetal death [120].
It has been suggested that convincing evidence should be present in order to conclude that an umbilical cord complication is the likely cause of death. Other recognized causes of stillbirth should be excluded through a careful and systematic evaluation and there should be evidence of cord occlusion and hypoxia on perinatal postmortem examination and histological examination of the placenta and umbilical cord, as suggested by Parast [37].

1.1.4.8 Placental abruption

Placental abruption, classically defined as the complete or partial separation of the placenta before delivery, occurs in approximately 0.4-1% of pregnancies, with lower

figures in Nordic countries and higher in USA [128]. It is associated to one third of all perinatal death and can be a threat to the mother's life [128, 129]. It is a clinical diagnosis, although the symptoms are not well-defined; they include abdominal pain with abnormal uterine contractions, abnormal cardiotocography with signs of distress and vaginal bleeding. However, some women will have an internal/concealed abruption in which blood from placental separation remains trapped behind the placenta, never passing through the vagina, making the diagnosis and decision to interrupt the pregnancy more difficult to the clinician, especially in preterm gestational weeks. If placental abruption shall be considered as the cause of death there should be clinical signs of a large abruption and in these cases placental histology will seldom help. However in some cases of recent abruption the diagnosis can be confirmed or at least inferred to by the presence of adherent retroplacental clot or fresh intravillous hemorrhages. If the abruption has been of more chronic nature, placental histology is more informative: the retroplacental clot, sometimes creating the tell-tale impression on the maternal surface, is often more obvious and additional diagnostic signs may be seen, i.e. hemosiderin deposits or signs of reduced placental perfusion (e.g. increased syncytial knotting, infarction), in the adjacent placenta parenchyma. In general, though, histopathological diagnosis of abruption is considered difficult and there are no agreed-upon, clear cut criteria [130].

Even if a variety of risk factors to placental abruption are identified the precise mechanism and etiology remain, to a large extent, unclear. The most important recognized risk factors are smoking, hypertensive disorder, (prolonged) preterm premature rupture of membranes (P-PROM) and history of previous abruption [128, 129]. Suggested mechanisms are impaired placentation, placental insufficiency, intrauterine hypoxia and uteroplacental underperfusion. An association to acute inflammation caused by infection, most commonly in preterm gestations with P-PROM, is also proposed [129]; it is assumed to be related to vascular destruction secondary to placental inflammation.

1.1.4.9 Preeclampsia/Hypertensive diseases

Preeclampsia, especially eclampsia, is one of the most important causes of maternal mortality globally; in developing countries a quarter of stillbirth and perinatal death may be associated to preeclampsia/eclampsia [131]. Hypertensive disorders (chronic hypertension and preeclampsia) are associated to stillbirth as a result of the association to placental insufficiency, IUGR and abruption [132]. Especially early onset preeclampsia (before gestational week 34) has been related to abnormal placentation leading to placental insufficiency and IUGR [133]. It is unclear whether milder or late-onset hypertensive disorder (without complication as placental insufficiency, IUGR and abruption) confers significant increase in the risk for stillbirth.
The risk of stillbirth has been shown to increase if the mothers develop HELLP-syndrome (hemolytic, elevated liver enzyme, low platelet), those women also have an elevated risk for eclampsia [134]. Eclampsia can be the cause of death of both mother and fetus due to i.e. hypoxia or other mechanisms [135].

1.1.4.10 Diabetes mellitus

Women with diabetes mellitus (both type1 and 2) are about five times more likely to succumb to stillbirth, especially if the glycemic control is poor [136]. The majority of stillbirths in diabetic women are related to complications of macrosomia, polyhydramniose, IUGR and preeclampsia [137]. The exact mechanism for fetal death in diabetes is uncertain, but maternal hyperglycemia is presumed to cause fetal hyperglycemia and hyperinsulinemia leading to accelerated fetal growth. The increased fetal metabolism is associated with chronic fetal hypoxia [136]. In addition, maternal vasculopathy and hyperglycemia can cause a decrease in uteroplacental perfusion [138]. Population studies have consistently shown a two- to six-fold increase in major malformations in infants born of diabetic mothers. Although the precise mechanism leading to fetal malformations is poorly understood, it has long been established that poor glycemic control at time of conception, duration of disease and the presence of vascular co-morbidities are associated with the risk of malformations. [136].

1.1.4.11 Intrahepatic cholestasis of pregnancy (ICP)

Intrahepatic cholestasis of pregnancy (ICP) is the most common liver disease in pregnancy and characterized by pruritus and elevated serum bile acids (≥10 μmol/L) and/or elevated liver transaminases, with onset in the second half of pregnancy and persisting until delivery [139]. ICP can have severe consequences for the fetus and is associated with an increased risk of spontaneous pre-term labor, fetal distress and stillbirth [140]. The risk of adverse fetal outcomes is reported to be increased in pregnancies where the maternal bile acid levels exceed 40 μmol/L [139]. The pathological mechanism underlying ICP-related fetal distress, preterm labor and stillbirth are not fully understood, but bile acids have been shown to cause vasoconstriction of isolated human chorionic plate veins, possibly resulting in a hypoxic environment for the fetus [141]. Two recent studies have shown ICP affected placentas to have a higher number of syncytial knots, which supports the theory of bile acid causing hypoxia [140, 142].

1.1.4.12 Other causes of stillbirth

The causes of stillbirth briefly discussed above are not a complete list of the causes proven or suspected to cause stillbirth; additional causes that may be considered are, for example, other maternal illness, i.e. coagulation disorder, antiphospholipid syndrome, renal and thyroid disease; complications due to multiple gestations as twin-to-twin transfusion; or, uterine rupture [37, 137].

1.1.5 Stillbirth in different gestational age

Few studies have investigated how causes of stillbirth differ over gestational ages. The existing studies show varying results, and are hard to compare because different classifications are used [11, 22, 24, 143]. Several studies have shown stillbirths before 28 gestational weeks to be commonly associated with infection [11, 22, 143] and malformations [144]. Previous studies of causes of stillbirth in different gestational ages have not included stillbirth in post-term pregnancy as a separate group for analysis [11, 22, 24, 143].

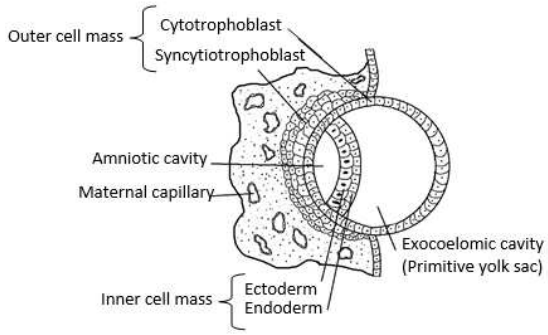
Fetal morbidity and mortality are increased in post-term pregnancy [145, 146], where among other factors macrosomia, meconium aspiration, and delivery trauma are over-represented. Further investigation concerning cause of death is needed in this vulnerable age group. In Sweden post-term pregnancy is defined as ≥ 42+0 weeks of gestation and the frequency was in 2009 approximately 4% [2]. The clinical praxis in Stockholm concerning induction of post-term pregnancies was changed in 2005 from gestational age 43+0 to 42+0. Risk of antepartum stillbirth has been shown to increase in the late term and post-term gestational weeks, from one per 2000 pregnant women at 37 gestational weeks to one per 500 pregnant women at 42 gestational weeks [147]. In a recent study, including stillbirth between gestational week 20+0 and 42+0, the authors shown a U-shaped relationship with the lowest stillbirth rate for fetus at risk in gestation weeks 25 to 28 [148].

1.2 HUMAN PLACENTA

Placenta, the latinized version of the greek word πλακουντας (plakountas), originally denoting discoid cake, is the only organ made up of cells originated from two different organisms. It is a complex organ with a short biological existence.
During fetal life the placenta replaces fetal organs as lungs, gastrointestinal, endocrine glands and kidneys and hereby maintains the feto-maternal vital functions of exchange of gases (i.e. oxygen and carbon dioxide), nutrients (i.e. glucose, amino acids, lipids), hormones (i.e. estrogens, progesterone and human chorionic gonadotropin) and waste products [149, 150].

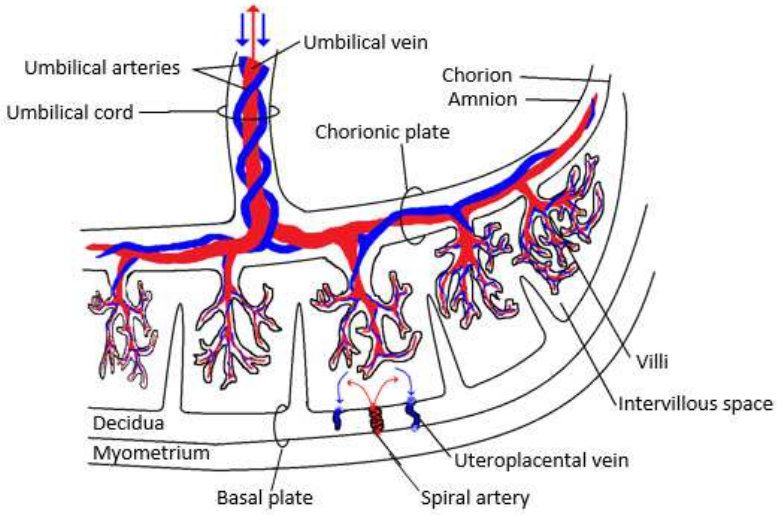
The fertilized human ovum undergoes a series of mitotic divisions to become a morula and then a blastocyst where the cells are arranged in an inner and outer cell mass. The inner cell mass gives rise to the embryo, umbilical cord and the placental mesenchyme, the latter is derived from the extraembryonic mesoderm. The outer cell mass will form the trophoblast that gives rise to large part of the placenta and fetal membranes [151, 152]. The embryo/inner cell mass is separated from the outer cell layer by a small cavity that during the pregnancy will enlarge to become the amniotic cavity and fuse with the chorionic layer, together surrounding the fetus and amnion fluid [153, 154], *Figure 1*.

Figure 1. *Schematic picture of late blastocyst.*



The amnion is composed of a single layered epithelium and the amniotic mesenchyme, an avascular connective tissue [152]. The blastocyst implants into the endometrium, which after the implantation will be transformed to become the decidua. During the blastocyst implantation, the trophoblast part differentiates into two layers, an inner cytotrophoblast and an outer syncytiotrophoblast, that invade the decidua and form primary stem villi. The primary stem villi develop further into a tree-like structure, to secondary and tertiary stem villi. The villous internal core will be penetrated by mesodermal cells differentiating into the villous capillary system. The tertiary villous capillaries end in the intervillous space where they are surrounded by maternal blood. In the intervillous space the feto-maternal exchange of oxygen, nutrients etc. will be performed. The blood then goes back to the fetus through the villous, chorionic plate and umbilical vein and returns to the tertiary villi through the two umbilical arteries, chorionic plate and villous vessels and so on. The maternal blood gets to the intervillous space through the spiral arteries (passing through the uterus myometrium and the decidua) and transfers back to the maternal circulation by uteroplacental veins (*Figure 2*) [150, 155-157].

Figure 2. *Schematic picture of human placenta.*



To effectively deliver blood to the intervillous space the spiral arteries are transformed in early pregnancy. This transformation is achieved by the trophoblast invasion that replaces the muscle- and elastic parts of the spiral arteries changing them to distended flaccid vessel. If the trophoblast invasion fails there will be an inadequate placentation, a feature underlying such severe pregnancy complications as preeclampsia and IUGR [150, 155].

The human placenta consists of a fetal part (chorionic plate, villi, umbilical cord and fetal membranes) and a maternal part (decidua) with two separate blood circulations. Until about mid pregnancy, maternal and fetal circulation is separated by four layers, constituting the so called placental barrier: the endothelium of the fetal capillaries, the connective tissue of the villi, the cytotrophoblast and the syncytiotrophoblast. As the

pregnancy advances the placental barrier becomes gradually thinner, mainly depending on the significant reduction of cytotrophoblast and connective tissue; in term pregnancy syncytiovascular membranes, only 4-5μm thick, are formed in terminal villi bringing maternal and fetal blood in close apposition. In recent years it has become apparent that the placental barrier is not absolute and even allows bidirectional cell traffic between mother and fetus [150, 155-158].

1.3 PLACENTAL INFLAMMATION

Different infectious agents are widely presumed to produce distinct patterns of inflammation in the placenta. The infection is thought to reach the placenta and fetus by two pathways; as an ascending infection from the lower genital tract to the fetal membranes and/or amniotic fluid and fetus or as a hematogenous spread of organism from the maternal blood stream to the placenta and fetus. Other less usual pathways are also suggested e.g. via the Fallopian tube, direct spread from the endometrium and iatrogenic during invasive procedures as amniocentesis [99, 159, 160].

The vagina is normally colonized by a wide range of bacterial species; these organisms and other pathogens do not usually cause clinically significant effects during pregnancy because of structural and functional barriers. These barriers are very important since the maternal immune system is partly down regulated to avoid rejection of the semi-allogeneic (immunologically foreign) fetus [161]. Structural barriers include the normally closed cervix canal with its mucous plug, integrity of the continuous chorioamnionic membrane and the syncytial nature of the outer layer of the villi (syncytiotrophoblast) [99]. Functional barriers include the endocervical secretory immune system, the expression of antibacterial proteins in endometrial glandular secretions and the amniotic fluid, as well as the rapid mobilization of myeloid lineage cells, derived from the mother and fetus to the critical maternal-fetal interface[162-165].
For example, entering of microorganisms through the cervix may result in stimulation of a maternal and fetal inflammatory process, initiating prostaglandin release that ripens the cervix, leading to labor and/or a clinical chorioamnionitis [159]. Clinical chorioamnionitis is not strictly defined; it includes a variety of symptoms, such as elevated maternal temperature, maternal tachycardia, fetal tachycardia, maternal leucocytosis, uterine tenderness, abdominal pain, foul smelling vaginal discharge, and elevated white blood cell count [159, 166, 167]. In the majority of cases the infection is subclinical, and specific diagnosis or confirmation is achieved following pathological examination of the delivered placenta and/or fetus [99].

Histological acute CAM is a maternal inflammatory response with acute inflammation of the placental membranes. Initially maternal neutrophils enter through the intervillous space spreading below the chorionic plate, leading to subchorionitis. This will be followed by membranous chorionitis, consisting of a thin row of neutrophils arrested at the interface between the decidua and the chorion layer. Thereafter the neutrophils spread to the amnionic connective tissue, and CAM is developed. A later stage is characterized by cellular death of both neutrophils and amnionic epithelium, manifest histologically by karyorrhexis of neutrophils and ischemic necrosis of the amnionic

epithelium (necrotizing CAM) [160]. Later, an additional **histological fetal inflammatory response (FIR)** develops, with fetal neutrophils in the walls of the vessels of the chorionic plate and/or umbilical cord (vasculitis) and thereafter in the Wharton jelly of the umbilical cord (funisitis) [160].

Histological CAM is usually due to a bacterial infection but other agents as fungi and mycoplasma have been suggested to be causative [160]. Several studies, using appropriate culture methods, have shown a high degree of concordance between histological CAM and the isolation of bacteria from the membranes [168, 169] With the use of supplementary molecular techniques (i.e., polymerase chain reaction; PCR) even more organisms have been identified [88-90, 170], but there still remains a considerable proportion of placentas with histological CAM where no bacteria will be demonstrated. If histological CAM (where no microorganism can be detected) is due to other causes than infection or just due to inadequate culture method is not clear. In a recent study of placentas from low risk term pregnancies, histological CAM was not significantly associated with infection, despite accurate microbiological cultures for anaerobic and aerobic bacteria (including Mycoplasma and Ureaplasma species) but histological CAM was strongly associated with fever but also spontaneous onset of labor, longer lasting labor and rupture of membranes as well as a more "activated" inflammatory response (including high serum levels of interlukin-6 and interleukin-8) [171].

The frequency of histological CAM has been shown to be inversely related to gestational age in preterm placentas [172-175], in the two later studies including placentas from stillbirth a bimodal distribution of histological CAM was identified with higher incidences in the extremes of gestations [172, 174]. This may partly explain why the reported incidence of histological CAM is widely varying with incidences from 7% to 96% [19, 172, 174, 176-183], other explanations can be a wide variation of infection between developed and developing countries [85], differences in the definition of grading of CAM, small sample sizes, selection of material and inter-observer variability among reporting pathologists.

Fetal inflammatory response (FIR) is the hallmark of fetal inflammatory response syndrome (FIRS) in the placenta [184, 185]. FIRS was originally defined as an elevation of the fetal plasma interleukine-6 concentration [186], which was found to be a risk factor for short-term perinatal morbidity and mortality [184, 186] and for the development of long-term sequelae such as bronchopulmonary dysplasia and brain injury [184]. In contrast, FIR was significantly higher in infants who survived the neonatal period compared to those who died during the perinatal period [173]. One reason for surviving in the presence of FIR could be that FIR signals the onset of labor, a hypothesis suggested by Blackwell et al., in their study, which included 44 cases of fetal death [180] and in a recent work, Gordon et al., [172], found that FIR was significantly associated with spontaneous onset of labor but also that the absence of FIR was strongly associated with unexplained antepartal death. Additional support for this hypothesis is shown in a study that included 428 stillbirths, where the spontaneous onset of labor was more common in

fetuses with FIR [174]. The incidence of FIR in stillborn placentas differs between studies, with frequencies reported between 2% and 28% [172, 174, 178, 180-182].

A large variety of viral infections can lead to a chronic inflammation of the placental (villous) parenchyma, called **chronic villitis** [99, 161, 187]. Chronic villitis is defined as the presence of a mononuclear cell (lymphocyte, histiocyte, plasma cell) infiltrate in the villous stroma often with destruction/necrosis of the villous parenchyma [161, 187-190]. In contrast there is no or minimal involvement of fetal membranes and chorionic plate. Chronic villitis affects 5-15% of all third trimester placentas and has been associated mainly with IUGR, but also with adverse neurologic outcomes, stillbirth, premature delivery and high maternal BMI [191]. Chronic villitis in stillbirth placentas has been associated with increased detection of viral DNA, especially in advanced gestational ages, implicating a link between viral infection and the pathogenesis of stillbirth [187]. In the majority of cases of chronic villitis the etiology remains unknown, an entity often referred to as villitis of unknown etiology (VUE; [191]). VUE is believed to somehow represent an immune mediated process, similar to graft-versus-host disease and is reported to have a recurrence risk. Myerson et al. [192] demonstrated that the majority of lymphocytes in VUE are maternal T cells, and the process is associated to significant destruction of syncytiotrophoblast; this may contribute to the breakdown of the local placental barrier and the graft-versus-host-like invasion of maternal cells into the villi.

2 AIMS

The overall aims of this thesis were: to develop and validate a new classification of stillbirth and, by using the classification, analyze if causes of death differ at different gestational ages; and, to compare placental pathology particularly regarding signs of infection/inflammation, one of the most common causes of stillbirth, in a prospective case-control study.

Specific aims were:

- To develop a new classification of stillbirth during audit work. The classification allows for more than one cause of death, where the primary cause of death identifies the factor that initiated the chain of events leading to death. The causes of death have distinct definitions that grade the causes in definite, probable and possible association of stillbirth (paper I).
- To study a cohort of all singleton stillbirths' cases from major Stockholm area 1998-2009 regarding primary and associated causes of death in relation to gestational ages (paper II); primarily comparing preterm stillbirths (gestational week 22+0 to 36+6) with term/post-term stillbirths (gestational week ≥ 37+0) and further analyze differences between extremely preterm (gestational age 22+0-28+6) and moderately preterm (gestational week 29+0-36+6) as well as between term (gestational week 37+0 to 40+6) and post-term stillbirths (gestational week ≥ 41+0).
- To elucidate differences in histopathological findings between placentas from term stillborn cases and term liveborn controls in a prospective case-control study. Main focus in the study was signs of infection/inflammation: acute chorioamnionitis, vasculitis, funisitis and chronic villitis (paper III).
- To elucidate differences in histopathological findings between placentas from (prospectively collected) early preterm stillborn cases and (retrospectively collected) early preterm liveborn controls in a case-control study. Main focus in the study was signs of infection/inflammation; acute chorioamnionitis, vasculitis and funisitis (paper IV).

3 MATERIALS AND METHODS

3.1 STILLBIRTHS IN STOCKHOLM

Since 1998, a group of obstetricians representing all delivery wards in Stockholm and perinatal pathologists in Stockholm, the "Stockholm Stillbirth group", has regularly performed clinical audits of all stillbirths in the major Stockholm area. Stillbirth is defined as delivery of a fetus at gestational week ≥ 22+0 without evidence of life (e.g. Apgar score 0-0-0). Death can have occurred antepartum or intrapartum. All stillbirths in the area are investigated according to a consensus structured test protocol that was validated in an earlier study [15]. The protocol includes: maternal blood tests (i.e., hemoglobin concentration (Hb), coagulation tests, etc.) and some immunological tests; culture from the amnion and fetal heart blood; PCR analysis (CMV, Parvovirus B19 and Enterovirus in placenta); chromosomal analysis in amnion or placenta; placental examination and autopsy. The results together with relevant family, medical and obstetrical history are registered in a database (www.iufd.se), created by members of the Stockholm Stillbirth group. The database includes all cases of stillbirths with gestational age ≥ 22+0 delivered at any of the delivery wards in Stockholm since 1998. Cause of death is ascribed to each individual case according to the Stockholm classification of stillbirth (paper I and II). The Stockholm classification of stillbirth has been developed during audit work by the Stockholm Stillbirth group and the classification was validated using 95 cases from 2005, where earlier consensus by the group was considered as reference, by six investigators (three familiar and three unfamiliar with the classification; paper I).

3.1.1 Study population

All cases of stillbirth in this thesis are identified from the database (www.iufd.se).

Between 1998 and 2009 there were in total 1198 stillbirth cases, 109 were from multiple pregnancies (four from triplet pregnancies and 105 from twin pregnancies) and 1089 were from singleton pregnancies. The 1089 singleton stillbirths were analyzed considering differences in cause of death between different gestational ages by dividing stillbirth cases into two main groups: preterm stillbirths (gestational age 22+0 to 36+6) and term/post-term stillbirths (gestational age ≥ 37+0). The preterm group was subdivided into extremely preterm stillbirths (gestational week 22+0 to 28+6) and moderately preterm stillbirths (gestational week 29+0 to 36+6), the term/post-term group was subdivided into term stillbirths (gestational week 37+0 to 40+6) and post-term stillbirths (gestational week ≥41+0).
The proportions of primary causes of death were compared between premature and term/post-term stillbirths, between extremely preterm and moderately preterm stillbirths, and between term and post-term stillbirths. The associated causes of death were then added to the primary causes and the same comparisons between the groups were performed as with the primary causes (paper II).

Between 2002 and 2005 tree hundred and seventy-seven women delivered 382 stillborn infants from singleton and multiple pregnancies. The 382 stillbirths were compared considering cause of death according to Stockholm stillbirth classification (paper I).

Between March 2002 and December 2005, there were 349 infants delivered stillborn in the major Stockholm area. Placentas from 306 of those stillbirths were collected prospectively. Forty-three cases were excluded because of twin pregnancy (n=23) or because the placenta was not sent to the Pathology Department (n=20). Of the 306 placentas from stillbirths 126 were from term- (paper III) and 180 were from preterm stillbirths (gestational week 22+0 to 36+6). One hundred and twenty-eight of the preterm stillbirths were from gestational week 22+0 to 32+6 (here called early preterm stillbirths). Sixteen of those were excluded because of fetal malformation and/or chromosomal abnormalities, leaving 112 placentas for investigation (paper IV).

During the study time the first term liveborn infant subsequent to the stillborn case (no matter preterm or term stillbirth), born in the same delivery ward, independent of delivery method, was recruited as control. A total of 273 term controls were thus prospectively included. The “control” mothers were asked to participate in the study after their delivery and were included after acceptance. The case and the recruited control were born on the same day in 89% of the cases (paper III).

3.1.2 Preterm controls/references (paper IV)

Preterm liveborn controls (paper IV) were recruited retrospectively from the database of the Department of Pathology, Karolinska University Hospital Huddinge (2002-2005), which during the study period received placentas from liveborn preterm infants from Stockholm South General Hospital, Karolinska University Hospital Huddinge and Karolinska University Hospital Solna. These three hospitals were routinely sending placentas for histological analyses if they came from pregnancies delivered before gestational week 33, whereas placentas from gestational weeks older than 32 weeks were only sent under special circumstances (e.g., severe preeclampsia or fetal hypoxia). By including exclusively placentas from deliveries prior to gestational week 33, we aimed to decrease a probable selection bias toward more "diseased" controls. Because the control group does not represent a "healthy" preterm population, it is termed the “reference group”.
For references at gestational week 32 we selected every second placenta from the database of the Department of Pathology, spanning the entire year to avoid potential bias because of any eventual seasonal distribution of placental findings (notably, the distribution of infections). For references at gestational weeks 23-31, all registered placentas were included. To obtain a proportional distribution for cases and references at the earliest gestational weeks, the inclusion period was extended to 2008. There were no singleton liveborn infants from gestational week 22 during the study period.
In total, 181 gestational week-matched reference placentas were selected initially, but to get a distinct biological border between cases and references we decided not to include liveborns that were not showing sufficient viability to be transferred to the neonatal intensive care unit (7 from gestational week 24 and 7 from gestational week

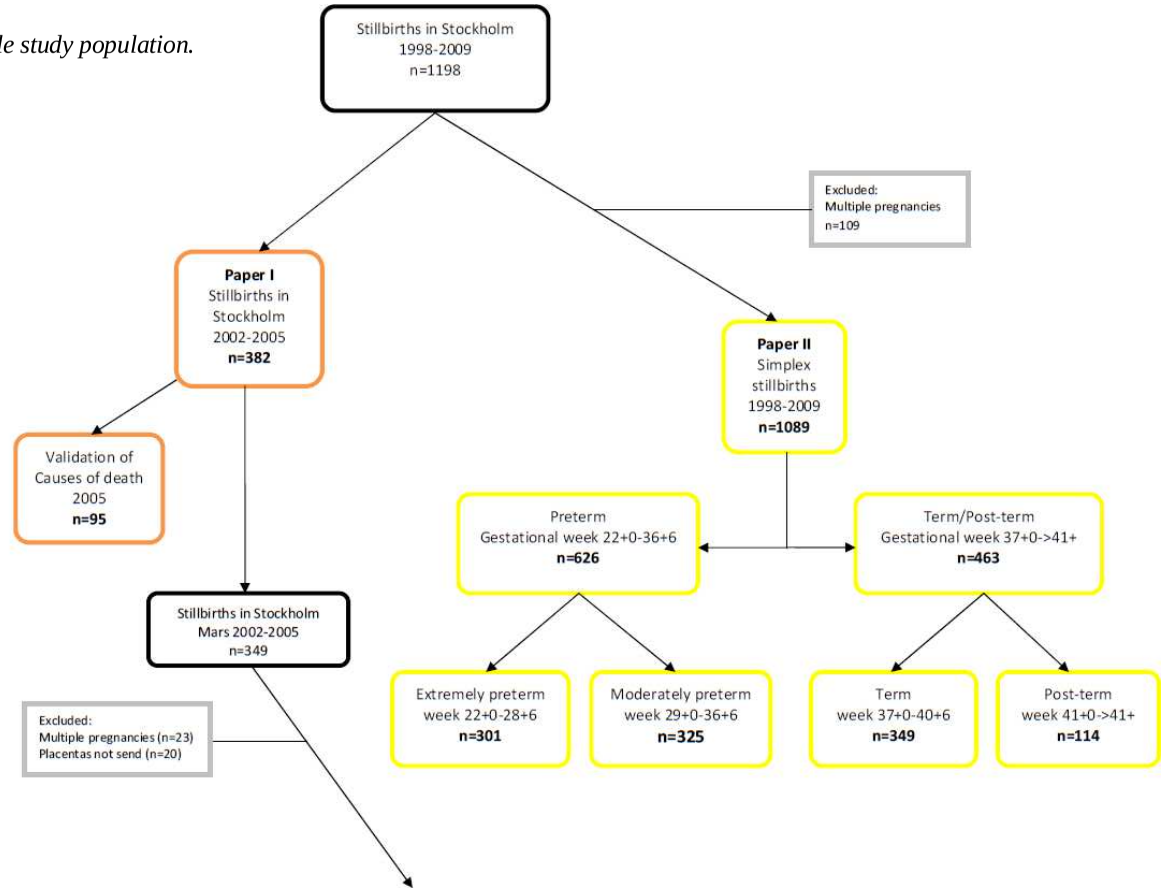
23); malformations/chromosomal abnormalities were additionally excluded (one infant with Trisomy 21), leaving in total 166 references.

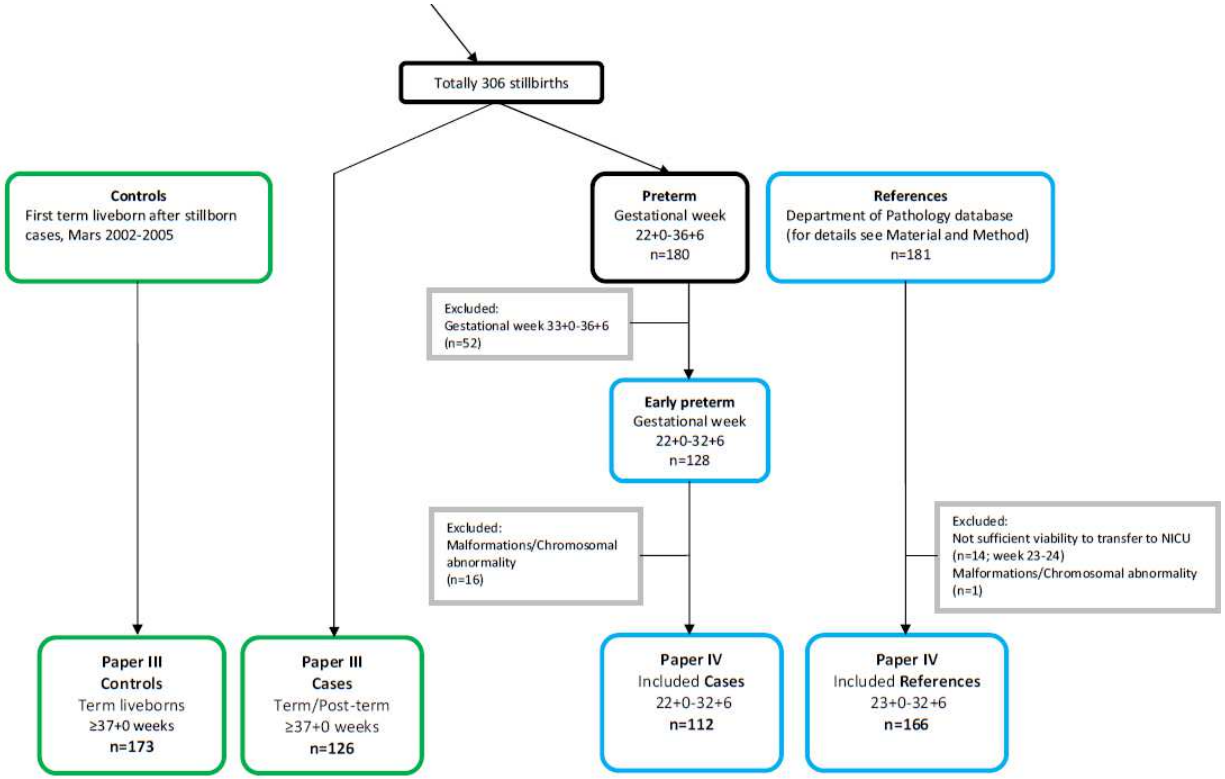
Flow chart for the whole study population is displayed in *Figure 3*.

All demographic data about the cases (paper I-IV) and term controls (paper III) were collected from maternal and delivery records registered in the database (www.iufd.se) whereas this information for the preterm references were collected from delivery records into an Excel-file (paper IV).

SGA was defined using the standard definition for SGA in Sweden, as birth weight < 2 standard deviations below mean [75].

Figure 3. *Flow chart for the whole study population.*





NICU, neonatal intensive care unit

3.2 HISTOPATHOLOGICAL EXAMINATION OF THE PLACENTAS

All placentas were sent to the Department of Pathology at Karolinska University Hospital, Huddinge. One senior perinatal pathologist performed all the histological investigations. This design was specifically chosen in order to avoid inter-observer variability in interpreting placental pathology [193]. Due to logistic circumstances the pathologist was aware of which group the placenta belonged to. The placentas were received fresh and were subjected to thorough gross morphologic assessment. The trimmed placental weight (after fixation) was compared to the gestational age and was determined to be low (<10th percentile), normal or high (>90th percentile), as previously described by Vinnars et al. [194]. For histological examination, we routinely included two samples from the cord, the membranes and two to three samples from macroscopically normal parenchyma as well as from any sites of focal change. Macroscopic and histological examination was performed according to a structured protocol (see appendix, paper III).

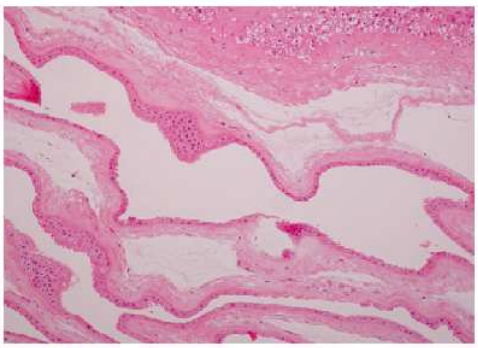
Placental data included in paper III and IV are: placental weight and low placental weight for gestational age, immaturity of placental villi (paper III) or accelerated villous maturation (paper IV), intervillous thrombosis, infarction, fetal thrombosis, presence of CAM, vasculitis in the placenta or umbilical cord, funisitis (inflammation of the umbilical cord) and chronic villitis. Since histopathological analysis cannot with certainty discriminate between fetal thromboses occurring before fetal death and those associated to post mortem changes this finding was only compared according to its frequencies in cases and controls/references but was not included in the logistic regression analyses.

CAM was defined as the presence of polymorphonuclear leukocytes in the chorion or amnion layers with or without membrane necrosis and was initially assessed using a three-grade scale, as described by Rindsjö et al. [195].

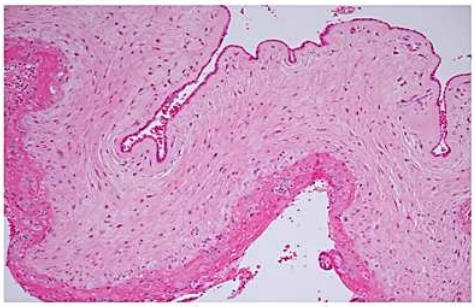
Grade 1: Presence of polymorphonuclear leucocytes at the subchorionic plate and/or lower third of chorion.
Grade 2: At least two separate foci of leucocytic infiltration in chorion and amnion.
Grade 3: Extensive leucocytic infiltration in chorion and amnion, together with necrosis of amniotic epithelium and/or microabscess formation.

Because of the very small amount of Grade 3 cases in paper III, Grade 2 and 3 groups were studied together, referred to as severe CAM, in the statistical analysis. For a histological picture of normal fetal membranes and CAM Grade 1-3 see *Figure 4*.

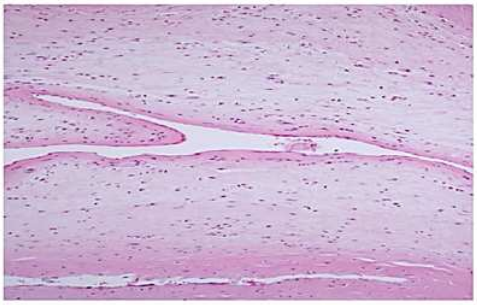
Figure 4. *Normal fetal membranes and acute histological chorioamnionitis, grade 1-3*



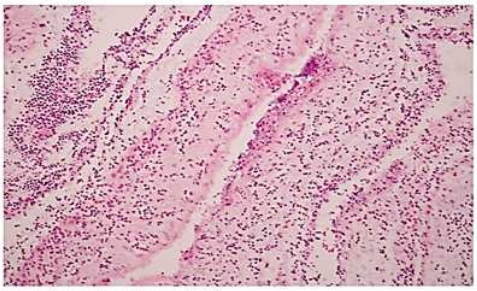
Normal fetal membranes.



Chorioamnionitis grade 1 (subchorionitis/chorionitis).



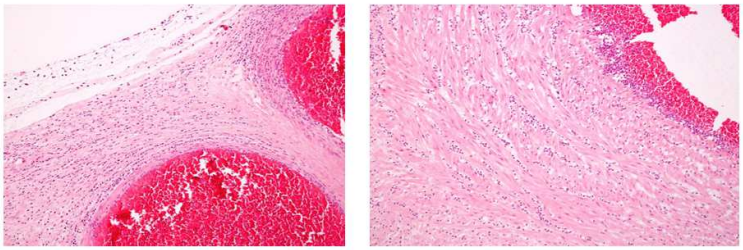
Chorioamnionitis grade 2.



Chorioamnionitis grade 3.

Vasculitis was defined as the presence of polymorphonuclear leukocytes in the vessel wall of chorionic plate or umbilical vessels (*Figure 5*). Funisitis was defined as the presence of polymorphonuclear leukocytes in the Wharton´s jelly. In paper IV, vasculitis and funisitis were studied together referred to as FIR.

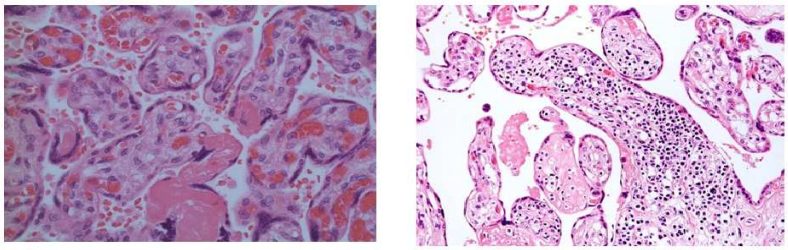
Figure 5. *Vasculitis in the vessel of chorionic plate and umbilical cord.*



Vasculitis in chorionic plate vessel. Vasculitis in umbilical cord vessel.

Chronic villitis was defined as the presence of a mononuclear cell (lymphocyte, histiocyte, plasma cell) infiltrate in the villous stroma, often with destruction/necrosis of the villous parenchyma (*Figure 6*).

Figure 6. *Normal villi in 3rd trimester and chronic villitis.*



Normal villi Chronic villitis

3.3 STATISTICS

Statistical analyses were performed using Statistica software, v. 8.0, StatSoft, Tulsa, USA and SPSS Statistics 17.0. A *p*-value <0.05 was considered statistically significant.

To assess the degree of agreement between investigators (paper I), kappa analysis was performed. Kappa values for agreement can be interpreted as almost perfect: ≥ 0.80; substantial: 0.60-0.79; moderate: 0.40-0.59; fair: 0.21-0.39 and poor ≤ 0.20 [196]

Bivariate analyses of categorical data were computed by univariable logistic regression and risk factors were expressed as odds ratios (OR) with 95 percent confidence intervals (95% CI). Statistically significant and borderline significant variables in the univariable logistic regression were entered in a stepwise forward multivariable logistic regression analysis to obtain adjusted odds ratio (AOR) with 95% CI (paper III-IV).

Table 4. Overview of the different statistical methods used in each paper.

	Student's t-test	Mann-Whitney U-test	Chi-square test	Fisher's exact test	Fleiss kappa statistics	Univariable logistic regression	Multivariable logistic regression (stepwise forward)
Paper I					x		
Paper II	x		x	x			
Paper III	x	x	x	x		x	x
Paper IV		x	x	x		x	x

3.4 ETHICS

All studies were approved by the Regional Ethics Committee of the Karolinska Institutet in Stockholm, Sweden.
D-nr: 97-440, 97-440B (paper I-II) and 2012/1706-32 (paper II)
D-nr: 02-012 (paper III-IV), 2008/748-32 (paper IV)

4 RESULTS

4.1 PAPER I

The Stockholm classification of stillbirth is exclusive for stillbirths from gestational week ≥ 22+0 and consists of 17 groups of cause of death. It allows for one primary and several added associated causes if needed. The primary cause of death is thought to identify the underlying cause of death, i.e. the condition or factor most likely to have initiated the chain of events leading to death. The causes of death are subdivided according to probability level (definite, probable and possible) by using definitions. The use of probability levels allows the classification to include causes of death that are individually too weak or too scarce to result in a definite association to cause of death. In our classification "Preeclampsia" includes preeclampsia as well as chronic- and pregnancy-induced hypertension. The group of cases with an unknown cause of death is in our classification divided into two groups: "Unknown" and "Unexplained". "Unexplained" is defined as: No positive findings in the investigation. "Unknown" is defined as: Obvious findings, but no definite association to stillbirth. The classification is primarily created to be used by audit groups. The Stockholm classification of stillbirth is presented in Table 5.

Table 5. The Stockholm classification of stillbirth.

1. Malformations and chromosomal abnormalities

Definite association to stillbirth

- Malformation or chromosomal abnormality that may cause stillbirth

Probable association to stillbirth

- Chromosomal abnormality in the placenta, combined with significant placental pathology that may cause stillbirth

Possible association to stillbirth

- Chromosomal abnormality in the placenta that may cause stillbirth
- Clinical significant malformation that according to the literature may cause stillbirth

2. Infection

2.1 Bacterial

Definite association to stillbirth

- Signs of infection in the fetus (e.g. pneumonia)
- Positive culture of heart blood or amnion or of maternal blood, combined with signs of infection in the placenta, i.e. subchorionitis/chorioamnionitis or vasculitis/funisitis

Probable association to stillbirth

- Clinical signs of chorioamnionitis in the mother, plus histopathological examination of the placenta showing subchorionitis/chorioamnionitis or vasculitis/funisitis
- Positive culture from sites other than heart blood, amnion or maternal blood, plus histopathological examination of the placenta showing signs of subchorionitis/chorioamnionitis or vasculitis/funisitis
- Histopathological examination of the placenta showing subchorionitis/chorioamnionitis and vasculitis/funisitis

Possible association to stillbirth

- Subchorionitis/chorioamnionitis or vasculitis/funisitis in the placenta, without positive cultures
- Clinical signs only of chorioamnionitis
- Positive culture of heart blood or amnion only (possible contamination taken into account).

2.2 Virus

Definite association to stillbirth

- Positive PCR (for selected virus) in the placenta or fetal tissue, plus signs of viral infection in the fetus or histopathological examination of the placenta showing signs of viral infection (e.g. viral inclusions or villitis)

Probable association to stillbirth

- Signs of viral infection in the fetus or in the placenta without a positive PCR (for selected virus)
- Positive PCR (for selected virus) in fetal tissue

Possible association to stillbirth

- Positive PCR (for selected virus) in the placenta only

2.3 Other

- Parasites (e.g. malaria, toxoplasma), fungi, etc, where a probability assessment is carried out individually

3. Immunization

3.1 Erythrocyte immunization

Definite association to stillbirth

- Signs of severe immunization in the mother, plus signs of anemia in the fetus

3.2 Thrombocyte immunization

Definite association to stillbirth

- Post mortem of the fetus showing massive hemorrhages, plus positive findings of thrombocyte antibodies in the parents making immunization possible

Probable association to stillbirth

- Post mortem of the fetus showing hemorrhages, plus positive findings of thrombocyte antibodies making immunization possible, in the parents

4. Feto-maternal transfusion

Definite association to stillbirth

- Findings suggesting massive feto-maternal transfusion, exceeding 40 % of the fetal blood volume
- Findings suggesting feto-maternal transfusion, with a fetal Hb >1 % and signs of anemia in the fetus (e.g. pallor or a sinusoidal cardiotocography (CTG)

Probable association to stillbirth

- Positive fetal Hb (>1%) but corresponding to less than 40 % of the fetal blood volume, plus findings in the placenta (e.g. increased erythropoiesis or edema)

Possible association to stillbirth

- Positive fetal Hb (>1%), but corresponding to less than 40 % of the fetal blood volume

5. Twin-to-twin transfusion

Definite association to stillbirth

- Monochorionic twin with ultrasound or clinical signs compatible with transfusion syndrome, plus histopathological examination of the placenta showing anastomoses with possibility for transfusion syndrome

Probable association to stillbirth

- Monochorionic twin with ultrasound or clinical signs of transfusion syndrome, when perfusion study of the placenta has not been performed

6. Birth hypoxia

Definite association to stillbirth

- Shoulder dystocia, malpresentation or other severe birth trauma
- Biochemical or electronic fetal monitoring suggesting fetal hypoxia

7. IUGR/placental insufficiency

Definite association to stillbirth

- Histopathological examination of the placenta showing >30 % parenchymal loss, such as infarction or villitis
- IUGR verified by ultrasound or post mortem examination, plus histopathological examination of the placenta suggesting placental insufficiency (e.g. 15-30% parenchymal loss or a small placenta <10th percentile or maturation defect or chronic villitis)
- Fetal thrombotic vasculopathy

Probable association to stillbirth

- SGA plus Histopathological examination of the placenta suggesting placental insufficiency (see above)

Possible association to stillbirth

- Histopathological examination of the placenta suggesting placental insufficiency (see above) with a normal weight baby
- IUGR verified by ultrasound or post mortem examination without placenta pathology

8. Umbilical cord complication

8.1 Umbilical cord prolapse

- Clinical signs of umbilical cord prolapse

8.2 Rupture of a vessel in the umbilical cord or membranes

Definite association to stillbirth

- Ruptured vessel in the membranes or the umbilical cord plus signs of fetal hemorrhage, such as pallor or sinusoidal CTG

8.3 Reduced circulation in the umbilical cord

Definite association to stillbirth

- Umbilical cord with tighten knot or tighten cord around the body or cord with stricture plus 2/4 signs:

  -Histopathological examination of the umbilical cord showing signs of stasis at the site of the knot,

  -Post mortem of the fetus showing signs of hypoxia,

  -Findings of thrombosis in the umbilical cord or chorionic plate,

  -Ultrasound showing decreased blood flow

Probable association to stillbirth

- Umbilical cord with tight knot or umbilical cord around the body or umbilical cord with stricture plus 1/4 signs (see above)
- Abnormal length or coiling of the umbilical cord plus 2/4 signs (see above)

Possible association to stillbirth

- Umbilical cord with tighten knot or tighten cord around the body
- Deviant length or coiling of the umbilical cord plus 1/4 signs (see above)
- Umbilical cord stricture without other findings

9. Placental abruption

Definite association to stillbirth

- Clinical signs of a large abruption

Probable association to stillbirth

- Histopathological examination of the placenta showing extensive signs of abruption (>30 %)
- Clinical signs suggesting placental abruption

Possible association to stillbirth

- Histopathological examination of the placenta showing signs of abruption

10. Preeclampsia

Definite association to stillbirth

- Eclampsia
- Preeclampsia and placental abruption
- Preeclampsia and IUGR

11. Diabetes mellitus

Definite association to stillbirth

- Previously known (or clinically diagnosed at birth) type 1 diabetes and signs of intrauterine or intrapartum asphyxia
- Previously known (or clinically diagnosed at birth) type 1 diabetes and LGA or SGA
- Previously known (or clinically diagnosed at birth) type 1 diabetes and severe malformation

Probable association to stillbirth

- Type 2 diabetes or gestational diabetes and signs of intrauterine or intrapartum asphyxia
- Type 2 diabetes or gestational diabetes and LGA
- Type 2 diabetes or gestational diabetes plus histopathological placenta findings suggesting diabetes
- Type 1 diabetes with no other findings

Possible association to stillbirth

- Type 2 diabetes or gestational diabetes, with no other findings

12. Intrahepatic cholestasis of pregnancy (ICP)

Definite association to stillbirth

- Raised bile acid levels (>70 umol/L), plus a history of typical pruritus

Possible association to stillbirth

- Raised bile acid levels (40-70 umol/L), plus a history of pruritus

13. Uterine complication

Definite association to stillbirth

- Clinical signs of obstructed circulation due to uterine rupture or torsion

14. Coagulation disorders

Definite association to stillbirth

- Antiphospholipid syndrome diagnosed in the mother, plus histopathological examination of the placenta showing >30 % infarction or thrombosis in fetal placental vessels
- Antiphospholipid syndrome diagnosed in the mother and post mortem of the fetus showing thrombosis

Probable association to stillbirth

- Histopathological examination of the placenta showing infarction (15-30%) or thrombosis in fetal placental vessels plus raised levels of cardiolipin antibodies
- Diagnosed antiphospholipid syndrome
- Histopathological examination of the placenta showing >30 % infarction, plus antithrombin deficiency or homozygous factor V mutation

Possible association to stillbirth

- Diagnosed thrombophilia other than antiphospholipid syndrome plus histopathological examination of the placenta showing >15 % infarction or thrombosis in fetal placental vessels
- Medium raised levels of cardiolipin antibodies

15. Other conditions related to stillbirth*

For example:

- Trauma
- Asystole in the mother
- Malignancy in the baby or placenta
- AV block with SS-A and SS-B antibodies in the mother
- Storage disease in the placenta, e.g. glycogenosis
- Tumor in the placenta
- Preterm labor (PTL) in extreme prematurity

*Probability assessment is carried out individually

16. Unknown

- Obvious findings, e.g. hydrops, asphyxia and oligohydramnios, but no definite association to stillbirth

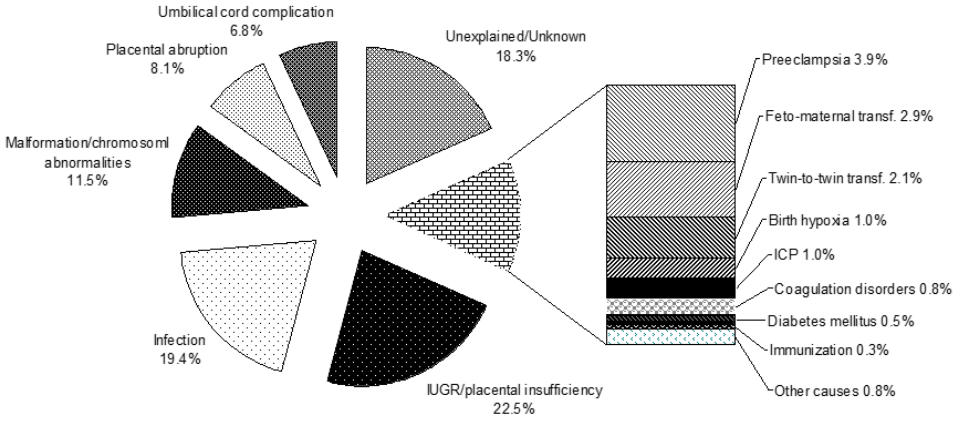
17. Unexplained

- No positive findings in the investigation

4.1.1 Primary death causes in Stockholm 2002-2005

During a four year period (from 1 January 2002 to 31 December 2005), 377 women delivered 382 stillborn infants at gestational week 22+0 or older. Twenty-six cases came from multiple pregnancies (all of those were twins). There were 382 primary causes of death and 132 associated causes of death registered. The leading primary cause of death was IUGR/placental insufficiency (86 cases; 22.5%), followed by infection (74 cases; 19.4%) and malformations/chromosomal abnormalities (44 cases; 11.5%). The unexplained group together with the unknown group comprised 70 cases (18.3%). The frequencies for all the groups are presented in *Figure 7*. In 42% the primary causes of death were definite, 17% were probable and 23% were possible.

Figure 7. *Primary causes of stillbirth 2002-2005.*



4.1.2 Validation of Stockholm Stillbirth classification

Ninety-five cases from 2005 were used for validation of the classification. In 89% of the cases, at least four of six investigators agreed about the primary cause of death. Agreement on the associated cause (number one and two, no case had more than two associated causes) was obtained by at least four of the six investigators in 79% (30/38) and 75% (6/8), respectively. The overall agreement for all investigators towards consensus was substantial according to kappa statistics (kappa: 0.70). The three investigators familiar with the classification showed agreement towards consensus (primary cause of death) in 95%, 90% and 86% respectively, whereas the figures for the three investigators unfamiliar with the classification were 80%, 76% and 74%

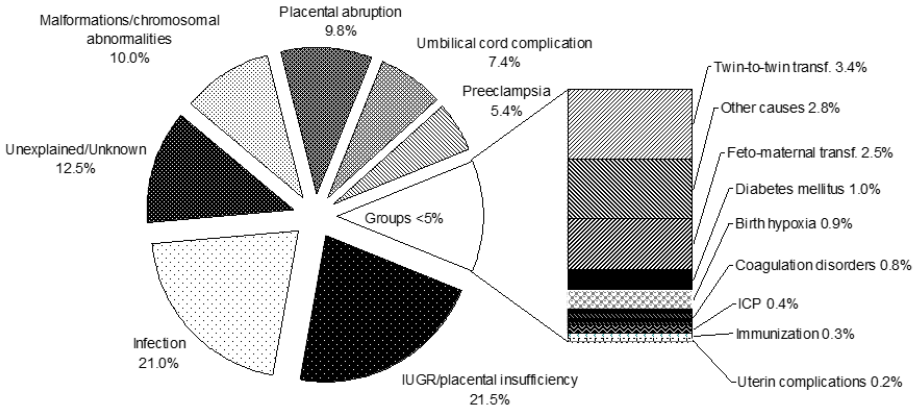
respectively. Overall agreement between investigators varied greatly regarding individual cause of death categories, for example twin to twin transfusion syndrome and malformations/chromosomal abnormalities showed almost perfect agreement (kappa:1.0 and 0.89 respectively), preeclampsia, IUGR/placental insufficiency, umbilical cord complication and infection showed substantial agreement (kappa:0.65, 0.64, 0.61 and 0.61 respectively), placental abruption showed moderate agreement (kappa:0.52) whereas "other causes" showed poor agreement (kappa:0.20).

4.2 PAPER II

4.2.1 Stillbirths in Stockholm 1998-2009

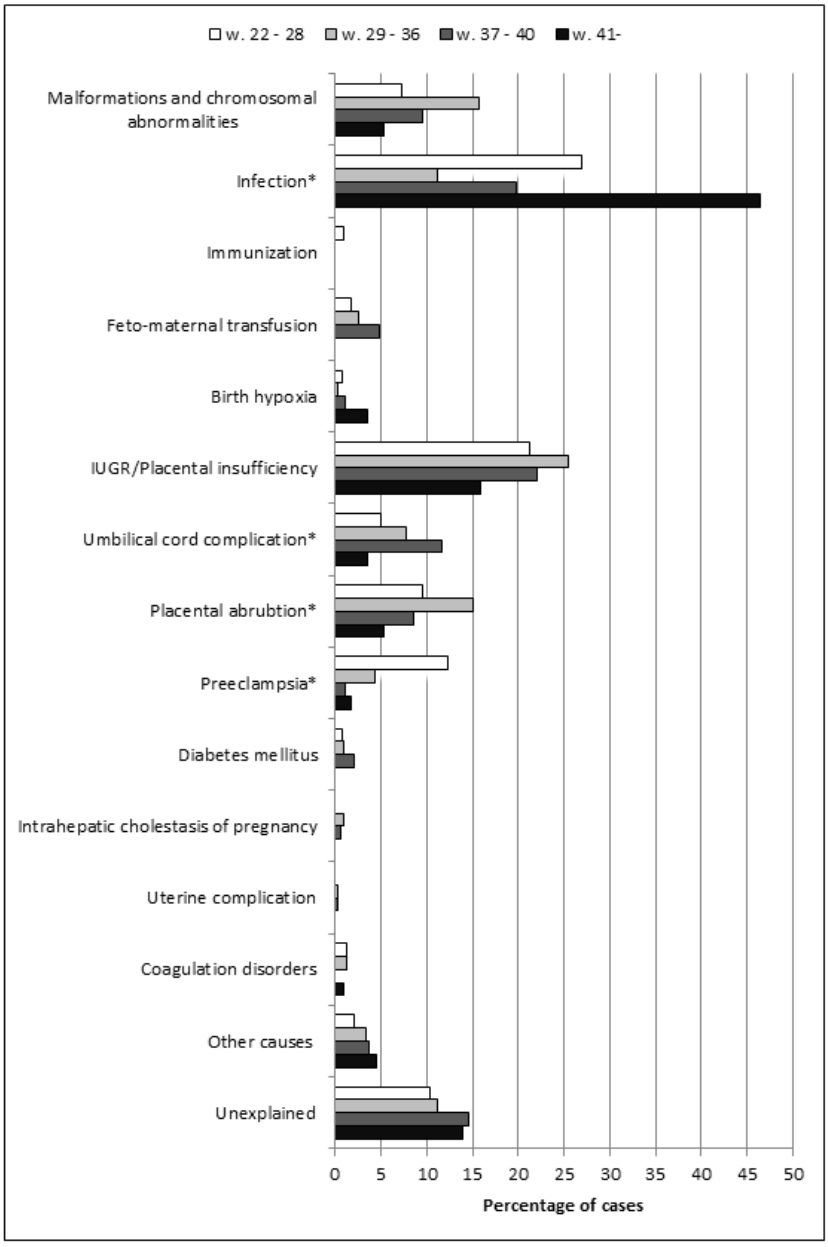
During a period of twelve years (from 1 January 1998 to 31 December 2009), there were 1198 cases of stillbirth from pregnancies ≥ 22 +0 weeks of gestation in Stockholm. The leading primary cause of death was IUGR/placental insufficiency (258 cases; 21.5%), followed by infection (252 cases; 21.0%), placental abruption (117 cases; 9.8%), malformations/chromosomal abnormalities (120 cases; 10.0%) and umbilical cord complication (89 cases; 7.4%). The unexplained group (together with the unknown group) comprised 150 cases (12.5%). Frequencies for all primary causes of death are presented in *Figure 8*.

Figure 8. *Primary causes of stillbirth 1998-2009*



In paper II the 1089 singleton stillbirth where compared according to primary cause of death and primary plus associated cause of death in different gestational ages. One hundred and nine stillbirth from multiple pregnancies were excluded (four from triplet pregnancies and 105 from twin pregnancies).
There were in total 1089 primary causes of death and 540 associated causes of death registered. The distribution of the causes of death in the singleton stillbirths were approximately the same as to the cohort of singleton and multiple pregnancies together; however the causes in the different gestational age group differed, se *Figure 9*.
For the distribution of singleton stillbirth in different gestational ages see *Figure 1*, paper II.

Figure 9. *Distribution of primary causes of death.*



* = significant difference in proportion when comparing groups of gestational week 22-36 and ≥ 37 w = gestational week.

4.2.2 Comparing preterm and term/post-term stillbirths

4.2.2.1 Demographic data

When comparing the 626 preterm (gestational age 22+0 to 36+6) and 463 term/post-term (gestational age ≥ 37+0) stillbirth cases, there were no significant differences in terms of mean maternal age, maternal age ≥35 years old, mean BMI, primiparity, prior stillbirth or sex of the stillborn infants. However, BMI ≥ 25 was significantly more frequent in the term/post-term group (45.4% vs. 38.8%). SGA was more common in the preterm group (45.9% vs. 17.0%) whereas large for gestational age was more common in the term/post-term group (5.5% vs. 2.6%), see Table 1 in paper II.

4.2.2.2 Primary causes of death

In preterm stillbirths, there were higher proportions of placental abruption (12.5% vs. 7.8%) and preeclampsia (8.1% vs. 1.3%). In term/post-term stillbirths, infection (26.3% vs. 18.7%) and umbilical cord complications (9.7% vs. 6.4%) were more frequent, Table 6. There was a tendency for more unexplained cases in the term/post-term group and for more malformations/chromosomal abnormalities in the preterm group, but the p-values did not reach significance level.

Table 6. Primary cause of death in preterm vs. term/post-term stillbirths.

Cause of death	All	w. 22 - 36	w. ≥ 37	
	n (%)	n (%)	n (%)	*p*-value
Malformation and/or chromosomal abnormalities	112 (10.3)	73 (11.7)	39 (8.4)	0.08
Infection	239 (21.9)	117 (18.7)	122 (26.3)	< 0.01
Immunization	3 (0.3)	3 (0.5)	0 (0.0)	0.27
Feto-maternal transfusion	30 (2.8)	13 (2.1)	17 (3.7)	0.11
Birth hypoxia	11 (1.0)	3 (0.5)	8 (1.7)	0.06
IUGR/placental insufficiency	242 (22.2)	147 (23.5)	95 (20.5)	0.27
Umbilical cord complication	85 (7.8)	40 (6.4)	45 (9.7)	< 0.05
Placental abruption	114 (10.5)	78 (12.5)	36 (7.8)	< 0.05
Preeclampsia	57 (5.2)	51 (8.1)	6 (1.3)	< 0.001
Diabetes mellitus	12 (1.1)	5 (0.8)	7 (1.5)	0.27
Intrahepatic cholestasis of pregnancy	5 (0.5)	3 (0.5)	2 (0.4)	1.00
Uterine complication	2 (0.2)	1 (0.2)	1 (0.2)	1.00
Coagulation disorders	9 (0.8)	8 (1.3)	1 (0.2)	0.09
Other causes	34 (3.1)	17 (2.7)	17 (3.7)	0.37
Unexplained	134 (12.3)	67 (10.7)	67 (14.5)	0.06
Total	*1089*	*626*	*463*	

w, gestational week; IUGR, intrauterine growth restriction

4.2.2.3 Primary and associated causes of death

When primary and associated causes where added together, the preterm stillbirths had a higher incidence of malformations or chromosomal abnormalities (12.9% vs. 8.9%),

IUGR/placental insufficiency (42.0% vs. 35.2%), placental abruption (24.1% vs. 11.4%) and preeclampsia (8.5% vs. 1.5%) compared to the term/post-term stillbirths. The term/post-term stillbirths had higher proportions of infection (39.5% vs. 29.2%), birth hypoxia (2.4% vs. 0.5%) and umbilical cord complications (18.8% vs. 13.1%), see Table 7.

Table 7. Primary and associated causes of death in preterm vs. term/post-term stillbirths.

Cause of death (primary and associated)	All	w. 22 - 36	w. ≥ 37	
	n (%)	n (%)	n (%)	*p*-value
Malformation and/or chromosomal abnormalities	122 (11.2)	81 (12.9)	41 (8.9)	< 0.05
Infection	366 (33.6)	183 (29.2)	183 (39.5)	< 0.001
Immunization	4 (0.4)	4 (0.6)	0 (0.0)	0.14
Feto-maternal transfusion	40 (3.7)	19 (3.0)	21 (4.5)	0.19
Birth hypoxia	14 (1.3)	3 (0.5)	11 (2.4)	< 0.01
IUGR/placental insufficiency	426 (39.1)	263 (42)	163 (35.2)	< 0.05
Umbilical cord complication	169 (15.5)	82 (13.1)	87 (18.8)	< 0.05
Placental abruption	204 (18.7)	151 (24.1)	53 (11.4)	< 0.001
Preeclampsia	60 (5.5)	53 (8.5)	7 (1.5)	< 0.001
Diabetes mellitus	20 (1.8)	11 (1.8)	9 (1.9)	0.82
Intrahepatic cholestasis of pregnancy	7 (0.6)	5 (0.8)	2 (0.4)	0.71
Uterine complication	2 (0.2)	1 (0.2)	1 (0.2)	1.00
Coagulation disorders	9 (0.8)	8 (1.3)	1 (0.2)	0.09
Other causes related to stillbirth	41 (3.8)	23 (3.7)	18 (3.9)	0.86
Unexplained [a]	134 (12.3)	67 (10.7)	67 (14.5)	0.06
Total primary and associated causes of death	*1618*	*954*	*664*	

w, gestational week; IUGR, intrauterine growth restriction

[a] Unexplained cases have only a primary diagnosis.

4.2.3 Comparing extremely and moderately preterm stillbirths

4.2.3.1 Primary cause of death

The extremely preterm stillbirths had higher proportions of infection (26.9% vs. 11.1%) and preeclampsia (12.3% vs. 4.3%) compared to moderately preterm stillbirths. The moderately preterm stillbirths had higher proportions of malformations or chromosomal abnormalities (15.7% vs. 7.3%) and placental abruption (15.1% vs. 9.6%), see Table 3 in paper II.

4.2.3.2 Primary and associated cause of death

When primary and associated causes where added together, the extremely preterm stillbirths had higher frequencies of infection (37.9% vs. 21.2%) and preeclampsia (12.6% vs. 4.6%) compared to the moderately preterm. The moderately preterm

stillbirths instead had higher frequencies of malformation or chromosomal abnormalities (17.2% vs. 8.3%), see Table 5 in paper II.

4.2.4 Comparing term and post-term stillbirths

4.2.4.1 Primary cause of death

The term stillbirths had higher frequencies of feto-maternal transfusion (4.9% vs. 0.0%) and umbilical cord complications (11.7% vs. 3.5%) compared to the post-term stillbirths. The post-term stillbirth cases had a higher proportion of infection (46.5% vs. 19.8%) compared to the term stillbirth cases, see Table 3 in paper II.

4.2.4.2 Primary and associated cause of death

When primary and associated causes where added together, the term stillbirths had a higher proportion of feto-maternal transfusion (5.7% vs. 0.9%) compared to post-term stillbirths, which had higher proportions of infection (57.9% vs. 33.5%) and birth hypoxia (5.3% vs. 1.4%), see Table 5 in paper II.

4.3 PAPER III

A total of 126 term stillbirth pregnancies were compared with 273 term liveborn pregnancies. Mothers of stillborn infants were older, had higher BMI and more often a history of miscarriage. The groups did not differ with respect to smoking habits, primiparity, previous stillbirth, severe illness or pregnancy complications, Hb at admission to maternity health care centre, sex of the infant or delivery method (i.e., caesarean section or vaginal delivery (Table 1(a) paper III). There was a higher rate of labor induction in the stillbirth group (Table 1(a) paper III). The stillborn infants were more often SGA (21.4% vs. 2.9%) see Table 1(a) paper III.
The stillborn group had a higher frequency of low placental weight for gestational age ($<10^{th}$ percentile) compared to the control group (50.8% vs. 33.5%; Table 1(b) paper III). Placentas from stillborn cases had significantly more CAM, villitis, immature villi, infarction, fetal thrombosis and vasculitis and/or funisitis; intervillous thrombosis did not differ between the groups (Table 1(b) paper III).
In the stillbirth group 65.1% had CAM (of any grade), compared to 40.7% in the control group ($p < 0.001$). CAM was initially graded (grade 1-3), but because there were only two cases of CAM grade 3 in the stillbirth group and none in the control group, CAM grade 2 and 3 were studied together (referred to as severe CAM), yielding a frequency of 33.3% in the stillbirth group and 11.4% in the control group. CAM grade 1 (referred to as light CAM) was found in 31.7% in the stillbirth group and 29.3%, in the control group (Table 1(b) paper III). Blood culture from the stillborns´ heart was performed in 102 (81.0%) of the 126 cases and was positive in 27 (26.5%) of the analyzed cases. There was no statistical correlation between positive bacterial culture of heart blood (positive in 27 of 102 cases) and CAM (independent of grade), vasculitis, funisitis, villitis or thrombosis (data not presented).

Chronic villitis was seen more frequently in the stillbirth group (18.3% vs. 5.1%). When sub grouping chronic villitis with respect to extent, chronic villitis < 1% was

found in 6.4% of the stillbirth group versus 1.8% of the control group and chronic villitis ≥ 1% was found in 11.9% in the stillbirth group versus 3.3% in the control group (Table 1(b) paper III).

Overweight or obesity (BMI > 24.9), history of miscarriage, SGA, low placental weight versus gestational age, immature placental villi, CAM (light and severe), vasculitis and/or funisitis and chronic villitis < 1% and ≥ 1% were all associated with an elevated risk for stillbirth in the univariable logistic regression. Smoking at registration in maternity centre, primiparity and severe illness or pregnancy complication, sex of the baby and intervillous thrombosis were not associated with an increased risk whereas age > 34 years and infarction of placental villi were of borderline significance (Table 2, paper III).

In the stepwise forward multivariable logistic regression severe CAM, immature placental villi and SGA had the highest risk for stillbirth, but even light CAM, villitis (< 1% and ≥ 1%) and BMI ≥ 24.9 were associated with an increased risk. Age > 34 years, previous miscarriage, low placental weight versus gestational age and infarction were not found to be independent risk factors and were therefore not included in the final model of the multivariable logistic regression. Vasculitis and/or funisitis did not either show an independent risk but was forced in the model since it has a well-known association with CAM [161, 190, 197] (Table 8).

Table 8. The adjusted odds ratio and their 95% confidence intervals for maternal and child demographic data and histopathological findings in placentas from stillborn cases and liveborn controls.

Variable	Adjusted OR	95% CI
BMI >24.9	2.06	1.21-3.51
Small for gestational age	7.52	3.06-18.48
Immature placental villi	7.17	2.66-19.33
Chorioamnionitis (graded)		
No	1	reference
grade 1	2.00	1.08-3.70
grade 2-3	7.39	3.05-17.95
Vasculitis and/or Funisitis	0.67	0.30-1.51
Villitis		
No	1	reference
< 1%	4.31	1.16-15.98
≥ 1%	3.87	1.38-10.83

CI, confidence interval; *OR*, odds ratio. BMI, body mass index.

4.4 PAPER IV

A total 112 preterm stillbirth cases were compared with 166 gestational week-matched liveborn references. There were no differences regarding the mother's age, gestational age, primiparity, previous stillbirth or infant gender (Table 1, paper IV). Chronic or pregnancy-induced hypertension was more common in the mothers of stillbirths (7.1% vs. 1.8%), while preeclampsia was more common in the mothers of reference infants (22.9% vs. 6.2%; Table 1, paper IV). SGA was more common in the stillborn group (50.9% vs. 30.3%; Table 1, paper IV). The rates of labor induction and Caesarean section were, as expected, different between the groups, with more inductions in the stillborn group and more Caesarean sections in the reference group (Table 1, paper IV). The median time between membrane rupture and vaginal delivery did not differ between the groups, but delivery > 6 hours after membrane rupture was more common in the reference group (Table 1, paper IV).

The stillborn group had a higher frequency of low placental weight for gestational age ($< 10^{th}$ percentile) compared to the reference group (54.0% vs. 32.9%; Table 2, paper IV). In the histological evaluation, there was no difference in intervillous thrombosis, infarction or chronic villitis (Table 2, paper IV). Accelerated villous maturation was more common in the reference placentas (Table 2, paper IV). Fetal thrombosis was, not unexpectedly, more common in stillborn placentas (51.4% vs. 10.2%; Table 2, paper IV).

The presence of CAM did not differ between the groups (33% in both groups), even if they were graded 1-3 (Table 2, paper IV). By contrast, signs of FIR were more common in the reference placentas (25.9% vs. 14.3%; Table 2, paper IV). All placentas presenting with FIR had CAM grade 2-3 (except for one case). When dividing the group with CAM into CAM with and without FIR, "CAM with FIR" was more common in the reference placentas (25.9% vs. 13.4%), while "CAM without FIR" was more common in the stillborn placentas (19.6% vs.7.2%; Table 2, paper IV).

Cultures from heart blood of the stillbirths were performed in 76 cases (67.9%). The cultures were positive in 31 cases (40.8%), but the laboratory suggested that approximately half of those cases were contaminations. There was no statistical correlation between positive bacterial cultures and CAM or FIR (data not presented).

Chronic or pregnancy-induced hypertension, SGA, low placental weight for gestational age and "CAM without FIR" were all associated to an elevated risk of stillbirth, while preeclampsia, accelerated villous maturation, FIR, vasculitis (in the placenta or umbilical cord) and "CAM with FIR" were associated to a reduced risk of stillbirth in the univariable logistic regression (Table 9). Maternal age $\geq$ 35 years, primiparity, previous stillbirth, infant gender, intervillous thrombosis, infarction, chronic villitis and CAM (regardless of FIR or no FIR) were not associated with an elevated or reduced risk of stillbirth in the univariable logistic regression (Table 9).

Table 9. The odds ratio for maternal and child demographic data and histopathological findings in placentas from preterm stillbirths and liveborn references**.**

Variable	*Tot n*	Stillborn n (%)	Liveborn n (%)	OR	95% CI
Maternal age ≥ *35 years*	*80*	28 (35.0)	52 (65.0)	0.73	0.43-1.25
Primiparity	*143*	56 (39.2)	87 (60.8)	0.91	0.56-1.47
Previous stillbirth (≥1 para)	*5*	3 (60.0)	2 (40.0)	2.18	0.35-13.49
Severe illness or pregnancy complication	*87*	30 (34.5)	57 (65.5)	0.70	0.41-1.18
Chronic or pregnancy induced hypertension	*11*	8 (72.7)	3 (27.3)	4.18	1.08-16.11
Preeclampsia	*45*	7 (15.6)	38 (84.4)	0.22	0.10-0.52
Infant gender (*Male)*	*139*	58 (41.7)	81 (58.3)	0.89	0.55-1.43
Small for gestational age	*104*	54 (51.9)	50 (48.1)	2.39	1.44-3.96
Low placental weight for gestational age	*114*	60 (52.6)	54 (47.4)	1.55	1.21-1.98
Accelerated villous maturation	*95*	24 (25.3)	71 (74.7)	0.36	0.21-0.63
Intervillous thrombosis	*57*	23 (40.4)	34 (59.6)	1.00	0.55-1.82
Infarction	*82*	37 (45.1)	45 (54.9)	1.33	0.79-2.24
Villitis	*15*	6 (40.0)	9 (60.0)	0.99	0.34-2.86
CAM	*92*	37 (40.2)	55 (59.8)	1.00	0.60-1.66
FIR (vasculitis and/or funisitis)	*59*	16 (27.1)	43 (72.9)	0.48	0.25-0.90
Vasculitis	*59*	16 (27.1)	43 (72.9)	0.48	0.25-0.90
Funisitis	*42*	13 (31.0)	29 (69.0)	0.62	0.31-1.26
CAM without FIR	*34*	22 (64.7)	12 (35.3)	2.71	1.27-5.81
CAM with FIR	*58*	15 (25.9)	43 (74.1)	0.52	0.27-0.99
No CAM	*186*	75 (40.3)	111 (59.7)	1	reference

CAM, acute chorioamnionitis; FIR, fetal inflammatory response;
CI, confidence interval; OR, odds ratio.

All variables in the univariable logistic regression that were associated with an elevated risk of stillbirth and “CAM with FIR” were analyzed in a stepwise forward multivariable logistic regression. Chronic or pregnancy-induced hypertension and low placental weight for gestational age were not independent risk factors and were not included in the final model of the multivariable logistic regression.
In the final model of the multivariable logistic regression, SGA and “CAM without FIR” were found to be independent risk factors of stillbirth, with an AOR of 2.13 and 2.44, respectively (Table 10).

Table 10. The adjusted odds ratio and their 95% confidence intervals from preterm stillbirths and liveborn references.

Variable	Adjusted OR	95% CI
Small for gestational age	2.13	1.26-3.62
No	1	ref
CAM without FIR	2.44	1.10-5.41
CAM with FIR	0.59	0.29-1.21
No CAM	1	ref

CAM, acute chorioamnionitis; FIR, fetal inflammatory response; CI, confidence interval; OR, odds ratio. The table displays results from the final model. Chronic or pregnancy induced hypertension and low placental weight for gestational age were not included because they were not found to be independent risk factors in the stepwise multivariate logistic regression; for further details see Methods.

5 DISCUSSION

5.1 MAIN FINDINGS OF THE STUDY

In this thesis we have developed a new classification for stillbirth, primarily intended to be used by audit groups (paper I). By using the classification we have compared causes of stillbirth according to gestational ages, mainly preterm versus term/post term, during a twelve year period in the major Stockholm area (paper II). The causes of death that most clearly differed between preterm and term/post-term stillbirths were preeclampsia (including even chronic- and pregnancy-induced hypertension) and placental abruption (more common in preterm stillbirths) plus umbilical cord complications and infections (more frequent in term/post-term stillbirths). The finding of infection being more common among term/post-term stillbirths was the most surprising, since infection is mostly connected with preterm stillbirths [11, 22, 85, 86, 143, 198]. When the preterm group was subdivided into extremely preterm and moderately preterm, and the term/post-term group was subdivided into a term and post-term stillborn, group infection was more common in the extremely preterm and post-term groups; although the relevant literature is conflicting, our results are in agreement with two recent studies of a bimodal distribution of CAM in stillbirth [172 174].

Infection is an important cause of stillbirth [37, 85-87, 199] and histological CAM is generally believed to represent an ascending microbial infection, mainly bacterial [160, 182, 183, 200], whereas virus is supposed to cause chronic villitis [99, 160, 161, 187]. Histological CAM has been shown to be clearly associated with stillbirth [85, 178, 181-183, 199, 201]. CAM, as well as FIR, is supposed to be a placental finding of bacterial infection [160], where FIR is the histological hallmark of FIRS in the placenta [184, 185]. FIR has also been shown to be associated with spontaneous start of labor [172, 174, 180]. In paper III, histological CAM, especially severe, and chronic villitis, were associated with an elevated risk of stillbirth in term pregnancies and in paper IV, histological "CAM without FIR", but not "CAM with FIR," was associated with elevated risk of stillbirth in early preterm pregnancies. SGA a well-known risk factor for stillbirth [3, 118], was in both paper III and IV associated with elevated risk of stillbirth.

5.2 HISTOLOGICAL CAM AND FIR

The incidence of histological CAM differs widely between studies [19, 172, 174-183], but is usually more common in stillbirth placentas when compared to controls [176, 178, 181-183, 201]. Several explanations have been proposed for the discrepancy of CAM; including different gestational ages [172-175], variation of the frequency of infection between developed and developing countries [85], small sample sizes, selection of material, differences in obstetrical practices as well as inter-observer variability among reporting pathologists. In an unpublished prospectively collected cohort (from Department of Laboratory Medicine, Karolinska Institutet) of 334 placentas from term uncomplicated pregnancies, the incidence of CAM was found to be 30% (grade 1: 13.2%, grade 2-3: 16.8%) [202].

In paper III, including term/post-term stillbirths and term live birth controls, the frequency of histological CAM was higher in the stillborn placentas, but when histological CAM was graded in light and severe the difference was only significant for severe CAM. This finding is not surprising, and could indicate that light CAM might be involved in the physiologic process of labor whereas severe CAM is probably due to a more severe or long lasting infection/inflammation [190, 193].

In a recent study on 1316 liveborn term placentas delivered after spontaneous onset of labor, histological CAM and funisitis were identified in 24% and 7% respectively and connected to longer duration of labor in multivariate logistic regression analyses. The authors made the interpretation that placental inflammation develops during the course of labor as some kind of physiological process [203]. In paper III we performed a sub analysis of half of the vaginally delivered cases and controls, considering duration of established contractions and the time interval between broken membranes and delivery (we only had data on this for half of the vaginally delivered mothers). We found that the controls had had a significant longer time from established contraction to delivery, ($P < 0.05$), but no difference in time period between broken membranes and delivery was observed (data not presented). Since we did not have data for the entire group, it is hard to draw any definite conclusions. However, in paper III histological CAM, especially severe CAM, was found to be an independent risk factor for stillbirth at term in the multivariate logistic regression analysis, which points towards an adverse outcome in term pregnancies when histological CAM is present. We also found FIR to be more common among the term/post-term stillborn compared to the term liveborn placentas, but in the multivariate logistic regression analysis FIR was not associated to an elevated risk of stillbirth. In total this leads to the conclusion that severe CAM, but not FIR, is a threat to the term fetus.

In paper IV, where the study group included early preterm stillbirth cases and early preterm liveborn references, the incidence of histological CAM was similar (30%) between the groups. This might initially seem surprising, but is easier to explain considering that preterm liveborn infants are hardly an uncomplicated, healthy group. The incidence of CAM in the early preterm liveborn placentas was in agreement with other studies from developed countries, where the histological definition of CAM was similar to ours [173, 175, 204]. Apart from our study (paper IV), we are not aware of any other case-control study of placental inflammation/infection exclusively using both preterm stillbirth cases and preterm liveborn controls.

FIR was in paper IV found to be more common in the early preterm liveborn reference placentas than in the stillborn placentas (25.9% vs. 14.3%). FIR has in two cohort studies of stillbirths from Australia [172, 174] been correlated to spontaneous onset of labor, and in the later study [172] the absence of FIR was strongly associated with unexplained ante partum death. In another study of a preterm cohort (gestational week 20-34) histological CAM and FIR were observed to be more common in preterm survivors of the neonatal period compared to perinatal deaths [173]. When we, in paper IV, divided the early preterm placentas with histological CAM into CAM with and

without FIR, we observed that “CAM with FIR” was more common among the early preterm liveborn references, whereas CAM without FIR was more common in the early preterm stillborn placentas. In the multivariate logistic regression analysis “CAM without FIR” was independently associated with a risk of stillbirth in early preterm pregnancies.
Our results indicate that the presence of CAM is not a risk factor for fetal death if FIR exists in early preterm pregnancies, but it seems to be a threat to the fetus if FIR does not occur. This can support the “rescue by birth theory”-hypothesis, i.e. that if an afflicted fetus can mount a sufficient immune response to trigger labor, it might be more likely to survive [180]. Our result, that FIR only occurred together with CAM grades 2–3 (except in one case), indicates that FIR does not appear until CAM is severe in early preterm pregnancies.

Another plausible explanation for the eventually protective effect of FIR (as compared to CAM only) for early stillbirth may be related to the release of dehydroepiandrosterone sulphate or cortisol [205], which may be beneficial to the fetus. Further, it may relate to the yet largely unexplored links between chorioamnionitis/infection and the regulation or reprogramming of the fetal/neonatal immune responses [206]. FIRS (in placenta identified as FIR, see background) involves the complex interaction of both pro- and anti-inflammatory cytokines and there is evidence that in many instances FIRS affected fetus are at risk for several complications, independent of gestational age at birth [206].

The question of whether CAM and FIR are caused by bacteria or is an immunologic process involved in labor or a response to other pathological processes, cannot be conclusively answered in this thesis. Cultures from heart blood of the stillbirths failed to show a bacterial etiology of placental inflammation in both paper III and IV. One reason for this finding might be that blood samples were only cultured for aerobic and anaerobic bacteria. Previous studies have questioned the efficacy of cultures alone for detecting bacteria, suggesting the need for supplementary molecular techniques (i.e., PCR) [88-90, 170]. The reason for the failure to show a bacterial etiology can also be due to that histological CAM and FIR might, at least to some extent, be non-infectious. Causes such as fetal hypoxia, amniotic fluid pH changes, and immunological responses to fetal tissues or meconium have been suggested but not proven [207]. In a recent study of placentas from low risk term pregnancies, histological CAM was not significantly associated with infection, despite accurate microbiological cultures. Instead the authors found histological CAM to be associated with fever, rupture of membranes, and spontaneous onset and longer lasting labor as well as a more “activated” inflammatory response. However, with only 15 cases of grade 2 histological CAM that study apparently was too small to assess association of grade 2 chorioamnionitis and infection [171]. On the other hand the study by Gordon et al. [172] of a cohort including 952 stillbirths found that the absence of FIR correlated with unexplained death. The study by Gordon used the PSANZ-PDC for classifying the cause of death, not including histological CAM as a definition for infection in stillbirth;

the authors suggested that undiagnosed infections can be a part of causes in these unexplained stillbirths with histological CAM.

Taken together, our studies indicate that histological CAM and FIR might have different etiology in preterm and term pregnancies. Furthermore, light (grade 1) and severe (grade 2-3) CAM might be responses to different causes. Light CAM can in term pregnancies be a physiological process, associated to the initiation of labor, whereas severe CAM is a pathological process in term pregnancies, mainly caused by infection. If a pathological process as infection initiates CAM, a preterm fetus that is able to mount a sufficient immune response (FIR) to trigger labor, might survive; this does not mean that the preterm infant will be healthy since it can be injured by, for example, cytokines that is involved in FIR. FIR has also been shown to be a risk marker for neurological impairments, such as cerebral paresis and decreased psychomotor development [190, 208].

5.3 CHRONIC VILLITIS

Chronic villitis was in paper III found to be more common in term stillbirth placentas than in term liveborn placentas (18.3% vs. 5.1%), and in the multivariate logistic regression analysis it showed, approximately, a four-fold risk for stillbirth whereas in paper IV it was not more common in stillbirth. Chronic villitis is in some cases due to an associated virus infection, most notably CMV infection. Syridou et al. [187] recently showed that the presence of chronic villitis in stillbirth placentas was associated with increased detection of viral DNA, especially in advanced gestational ages; implicating a link between viral infection and the pathogenesis of stillbirth. However, in the majority of cases, the etiology of chronic villitis remains unknown, often referred to as villitis of unknown etiology (VUE; [191]). Myerson et al.[192] demonstrated that the majority of lymphocytes in VUE are maternal T cells, and the process is associated with significant destruction of the syncytiotrophoblast. This may contribute to the breakdown of the local placental barrier and the graft-versus-host-like invasion of maternal cells into the villi.
Our data does not provide any clues regarding the underlying mechanism to chronic villitis. The higher frequency among the term compared to the preterm stillbirths we observed in the study might indicate that the preterm fetus cannot mount a sufficient immunologic response to a possible viral infection (or other stimulus) in early pregnancy.

5.4 SGA AND “IUGR/PLACENTA INSUFFICIENCY”

SGA is a known risk factor of stillbirth [3, 118]; the SGA group includes predominantly growth restricted infants, even if a significant proportion (30%) of SGA infants are not growth restricted [78]. In the Stockholm classification of stillbirth, SGA as an isolated finding cannot be considered as cause of death due to “IUGR/placental insufficiency”. Nevertheless, “IUGR/placental insufficiency” was the leading cause of death in the cohort of stillbirths in Stockholm 1998 to 2009. “IUGR/placental insufficiency” was, as a primary cause, as common in the preterm group as in the term/post-term group, but

when primary and associated causes of death were summated it was significantly more common in the preterm stillbirth group. Part of that difference can be due to the significantly higher amount of "preeclampsia" (that also include chronic- and pregnancy-induced hypertension) and "malformation and/or chromosomal abnormalities" as causes of death in the preterm group, even if the latter was only significantly higher when primary and associated causes of death were summated. However, stillbirth associated with IUGR, without any other obvious direct cause, is one of the major types of stillbirth [18, 77, 79].

SGA was found to be an independent risk factor of stillbirth in both paper III and paper IV. In paper II, SGA was a common finding (33.5%) in the total cohort of singleton stillbirths, but significantly more common in the preterm group (45.9% vs. 17.0%). Many preterm live births are also growth restricted [209]. In a population based study including singleton live births and stillbirths at 24-36 weeks gestation from the USA (n=902,491) and at 28-42 weeks gestation from Sweden (n=946,343), the difference in birth weight between live births and stillbirths increased with advancing gestational age. The authors suggested that growth restriction is a cumulative process and that preterm stillbirths grow more slowly before they die because of suboptimal placental function developing earlier in pregnancy [209]. The elevated risk of stillbirth in a group consisting of 50% SGA infants found in paper IV was not surprising, even though the liveborn group included 30% of infant with SGA.

SGA includes, as discussed earlier, infants with intrauterine growth restriction and may be related to maternal smoking and maternal illness, such as preeclampsia [210]. In paper III there was no difference in smoking habits (unfortunately we did not have this information in the reference group in paper IV) and we did not find an elevated risk of stillbirth among smoking mothers or mothers with severe illness or pregnancy complication. The lack of difference might be due to the small amount of smokers and mothers with severe illness or pregnancy complications in that study which might lead to a type 2 error, related to inadequate power for that analysis.

In paper IV, preeclampsia was more common among the liveborn references than the stillborn cases. This is not surprising, since the reference group in paper IV hardly consists of healthy infants. Pregnant women with preeclampsia undergo more frequent controls and get delivered when the risks regarding her health are considered too high. However, even if the reference mothers more often had preeclampsia, the stillborn mothers had instead more chronic and pregnancy induced hypertension which does not lead to the same frequency in controls as preeclampsia. SGA was more common in the stillborn group and an independent risk factor of stillbirth despite the higher frequency of preeclampsia in the reference mothers (Table 1, paper IV).

The high number of SGA infants, especially in the preterm groups of our studies, and the associated elevated risk of stillbirth, plus the high number of "IUGR/placental insufficiency" as a cause of death in both preterm and tem/post-term stillbirth groups, underline the importance of identifying those infants that are truly growth restricted in

order to be able to design better strategies for fetal surveillance and possible intervention in future pregnancies.

5.5 PREECLAMPSIA

In paper II, a higher proportion of preeclampsia was noted in the group of preterm stillbirths compared to the term/post-term stillbirth group; when the preterm group was subdivided most of stillbirths due to preeclampsia were identified in the extremely preterm group. When adding associated causes of death, preeclampsia remained more frequent in the extremely preterm group. These results concur with a study from the USA, showing stillbirth to be associated with hypertensive disorders, occurring more frequently in gestational week 24-31[22]. The clinical course of preeclampsia could provide some explanation for these results. When preeclampsia occurs in term pregnancy, delivery can be induced with minor fetal risk, thus minimizing risks of maternal and fetal complications of preeclampsia. In early-onset preeclampsia (before 34 weeks of gestation), the risk for perinatal complications with preterm delivery must be weighed against the fetal and maternal risks of preeclampsia, which include IUGR, placental abruption, eclampsia, and pulmonary edema [133, 211].
However, our study and the American study differed considering the total amount of stillbirth caused by preeclampsia, even though our definition of preeclampsia was quite similar to theirs (INCODE classification [39]). In our cohort approximately 5% of the stillborn cases were caused by preeclampsia whereas in their study approximately 9% were caused by preeclampsia. The reason for this discrepancy can be a higher amount of Afro-Americans, known to have a higher risk of preeclampsia, in their population [212]. Another explanation can be that the total incidence of preeclampsia in the USA has risen, which has been suggested to be related to an increased prevalence of predisposing disorders as chronic hypertension, diabetes and obesity [212]. The prevalence of some of these predisposing disorders appears to rise even in Sweden, albeit to a lesser degree. In 2009 almost 37% of mothers in Sweden were reported to have BMI $\geq$ 25 and 12% were obese [2] whereas in USA 2009-2010, 56% of women age 20-39 had BMI $\geq$ 25 and 32% were obese [213]. The lower frequency of preeclampsia in our cohort of stillbirths may additionally be associated with the more widely available specialized maternal care in Sweden.

5.6 PLACENTAL ABRUPTION

In paper II, placental abruption occurred more frequently in the preterm stillbirth group, both when exclusively analyzing primary causes, and when the associated causes of death were added to the primary causes. This finding stands in contrast to that of Bonetti et al. [23], who found placental abruption to be more common at later gestational ages, and with Copper et al.[143], who did not note a higher frequency of placental abruption at any specific gestational age. In the study by Bonnetti, the ReCoDe classification [18] was used. The ReCoDe classification identifies the relevant condition at the time of death as the primary cause and not, as in the Stockholm classification, the factor that initiated the chain of events leading to death. With this approach, in a fetus that dies because of abruption due to preeclampsia, the ReCoDe classification will assign abruption as the primary cause of death, whereas the Stockholm classification

would assign preeclampsia as the primary cause of death. This might partially explain some of the differences in the studies of primary causes of death.

Another possible explanation for placental abruption being more common in preterm stillbirths is that it is associated with P-PROM, and the risk of abruption increases with decreasing gestational age [128]. Theoretically, the sudden reduction of uterine volume when PROM occurs leads to abruption [214], but in contrast, women exposed to prolonged P-PROM who are managed expectantly are at increased risk of developing abruption if the time from membrane rupture to delivery exceeds 24 hours [128, 215]. The higher risk of abruption when P-PROM is managed expectantly can be due to the association with histological CAM, since both PROM and chorioamnionitis are associated with abruption [128, 216].

5.7 UMBILICAL CORD COMPLICATION

When comparing preterm and term/post-term stillbirths, umbilical cord complications were more frequent in term/post-term pregnancies. The Stillbirth Collaborative Research Network [22] noted umbilical cord abnormalities as a cause of stillbirth after 32 gestational weeks, and Ryan et al. [119] noted stillbirths due to cord accidents at later gestation, both of which are in line with our findings.

In the Stockholm classification of stillbirth, umbilical cord complication is a heterogeneous group including umbilical cord prolapse and rupture of unprotected vessel(s) in the membranes (vasa previa) because of velamentous cord insertion or accessory placental lobes. In these instances the association to stillbirth is obvious via acute or subacute hemorrhage. Another subgroup consists of conditions associated to reduced umbilical blood flow due to occlusion, entanglement or extreme umbilical cord length (both short and long) or coiling abnormalities. Abnormal length (especially short) may be difficult to document and is more likely to be associated with chronic umbilical flow compromise. Coiling pattern is established early in pregnancy and may be associated with other underlying vascular abnormalities in the placenta, additionally contributing to stillbirth, i.e. maturation defects [125, 127]. Stillbirths caused by acute umbilical cord complications, like umbilical cord prolapse, are unusual conditions and most likely represent a minority of these cases. The higher occurrence of umbilical cord complications in the term/post-term group in paper II is more likely to be caused by umbilical cord complications due to a more chronic or subacute nature (e.g. hypo- or hypercoiling and velamentous or marginal insertion of the cord), but also umbilical cord complications that can be due to both chronic and acute nature (e.g. true knots, tight cord around body or torsion), where hypoxia progressively worsens over time due to impaired circulation [120].

5.8 CLASSIFICATION OF STILLBIRTH

One might wonder why we have made a new classification when there were already more than 35 existing classification systems. However, we are not the only group who has made a new classification system in recent years. Four other groups in four different countries created, around the same time as us, four new classification systems,

resulting in: PSANZ-PDC from Australia / New Zealand [31], ReCoDe from Great Britain [18]; Tulip from Holland [10] and CODAC from Norway [38]. Later on, an American group also published a new classification system, INCODE [39]. The fact that we and many other groups clinically active in the field of stillbirth independently worked and published new classification systems during the beginning of 21st century is a strong indication that the existing classifications did not fulfill today's requirements. This was also shown in a recent evaluation by Flenady et al. [21], which included four of the new classifications (PSANZ-PDC, ReCoDe, Tulip and CODAC) and the two, probably most used, older classification systems (Amended Aberdeen [65] and Extended Wigglesworth [73]). According to the study results, the two older classification systems could not be recommended for use. This result is not surprising, considering that the two older classifications were constructed approximately 30-40 years ago, and both were in fact revisions of even older classification systems.

Many quality aspects must be taken into account when designing a classification system. A classification should: be able to identify the underlying cause of death (the factor that initiated the chain of events leading to death), illustrate associated factors which may have contributed to the death, be easy to use for clinicians, have a low inter-observer variability, be easy to expand in terms of sub-classification, be based on clinical factors and necropsy findings including histology of the placenta, result in a high percentage of classifiable cases, identify possibilities of reducing the perinatal mortality and have strictly defined criteria for each group in order to be able to compare different populations; some of these qualities will be discussed here.

The Stockholm classification of stillbirth has several similarities with the five other classifications from the 21st century, but also some important differences. Identifying the underlying cause is a common feature of all the new classifications (except ReCoDe which identifies the relevant condition at the time of death in utero). By having this design, and also allowing for more than one cause of death, the new classifications make it possible to reach a better understanding of the etiology of stillbirth which is important for achieving a reduction in stillbirth rates. Including more than one cause of death is an additional new important feature, since a number of conditions, rather than a separate entity, may have contributed to the death. Earlier classification systems mostly allowed for just one cause/association of death and solved the selection between different causes by applying (more or less) strict hierarchical systems to evaluate the significance of various findings; a device which could lead to missing a lot of important information. The classifications from the 21st century do not widely adopt this hierarchical approach; still, ReCoDe is mainly hierarchical (starts from conditions affecting the fetus and moves outwards in simple anatomic groups), whereas the Tulip and PSANZ-PDC classifications recommend the "hierarchical approach to be used as a guide" and INCODE uses the hierarchical approach when grading causes of death in present, possible, and probable cause of death.

The older classifications included a limited amount of causes of death. This approach is changed in "the 21st century classifications" and, for example, the relatively large amount of 17 cause of death-groups in the Stockholm classification might at first appear cumbersome or redundant. However, in most cases the findings will make the primary diagnosis quite obvious and much of the remaining work can focus on deciding

on the probability level and associated diagnoses or factors. When the classification was validated, none of the investigators (not even the three unfamiliar with it) experienced the classification as complicated. The other "21st century classifications" have fewer "major categories" but when including subcategories as well, CODAC will be the leading classification with 10 major causes, and 94 subcategories further specified in 577 subcategories. However, in the recent review of classifications by Flenady [21], CODAC performed the best considering information retention and easiness to use, suggesting that the use of many major- or subcategories is apparently an advantage for the classification system.

Except ease of use it is important that a classification has a low inter-observer variability. The Stockholm classification performed well (substantial) according to kappa statistics (kappa: 0.70) when overall agreement was evaluated for six investigators towards consensus, even if the agreement between the investigators varied greatly regarding individual diagnostic categories. Anyway, the most common causes as IUGR/placental insufficiency, infection and umbilical cord complication showed substantial agreement and malformation showed almost perfect agreement even if placental abruption just showed moderate agreement. The result from this evaluation leads to the conclusion that the Stockholm classification over all has a low inter observer variability. In the previously referred evaluation by Flenady et al. [21] the Tulip classification proved the best with a kappa value of 0.74, CODAC and PSANZ-PDC performed good with kappa value of 0.65 and 0.63 respectively and ReCoDe fair with kappa value of 0.51; the two older Amended Aberdeen and Extended Wigglesworth had poor agreement with kappa value 0.35 and 0.25, respectively.

It is also important that a classification results in a high percentage of classifiable cases and low percent of unclassifiable cases. In paper II, we found that 12.5% of the total cohort cases (including both singleton and multiple pregnancy during 12 years in Stockholm) were unclassifiable. This is in good agreement with 4 of the other new classifications showing unexplained stillbirth between 9.5% and 15.4% [21], whereas INCODE (from 2011) showed somewhat higher amount of cases with unidentified cause of death [22]. In contrast, the two older Amended Aberdeen and Extended Wigglesworth had much higher rates of unexplained cases, 50.2% and 44.3% respectively [21].

Classifications shall have strictly defined criteria for each cause of death group, in order to i.e. be able to compare different populations. In an earlier work by the Stockholm stillbirth group [15], we found that clearly defined criteria are necessary when deciding what diagnoses/conditions are related to stillbirth. Assignment of a probable cause of death is also important to develop interventions for stillbirth prevention. Defining criteria to the Stockholm stillbirth classification has not been easy but discussions in the audit group have been instrumental and the group has strived to formulate well-defined criteria for each condition; when the existing knowledge has not been adequate for defining clear criteria the group has relied on its collective empirical knowledge. This approach will of course confer subjectivity to the definitions. However, the approach using definitions will in the end be less subjective compared to the approach in most of the older classifications that do not have any definitions at all.

Some diagnoses/signs are obviously the causes of death, e.g. clinical signs of umbilical cord prolapse, preeclampsia with total placental abruption and anencephaly whereas others, such as bile acid levels on 50µmol/L in a pregnant woman, are more unclear. We addressed this problem by grading the cause of death ascribing the level of probability/certainty into: definite, probable, and possible, according to the signs/findings. This solution appears to confer additional subjectivity to the classification, but by grading the cause of death many etiologic factors or diseases contributing to the fetal death can be included. The grading approach raised a lot of interest during a workshop arranged by the "National institute of child health and human development" in 2007 where, among others, representatives from all the six 21st century classifications met to summarize issues surrounding causes of death for stillbirths. The workshop resulted in definitions of causes of death that, regardless of which classification of stillbirth used, shall be considered as the cause of death [37].

Our approach of defining causes of death has been used in a modified way in the INCOD classification, the latest of "the 21st century classifications". The INCOD classification was developed after the above mentioned workshop. In the INCODE classification, causes of death are subdivided into being: probable, where the condition has high likelihood of death; possible, defined as a condition that cannot with high likelihood be considered to have caused the death but with reasonable certainty involved in a pathophysiological sequence that led to the fetal death and; present, defined as a condition of interest that cannot be classified as probable or possible.

Considering if a classification shall include all perinatal deaths or only stillbirths the "21st century classifications" are divided into two groups. The Stockholm classification of stillbirth, as well as INCODE and ReCoDE, only include stillbirths whereas the others include perinatal death (and Tulip also includes "infant mortality"). We believe that causes/associations of death between stillbirth and neonatal death are basically different, even though there is some obvious biological overlapping, and that they should optimally be classified separately.

None of the "21st century classifications" are approved as an international consensus classification, nor was this the goal when we developed the Stockholm classification. Our goal was to develop a classification mainly for audit groups, with clear/strict definitions for causes of death used for stillbirth cases only. Careful investigation according to a structured test protocol [15] including, among other, autopsy and placental examination as well as a review of clinical history shall be used to optimize the use of the classification.

5.9 STRENGTHS AND LIMITATION OF THE STUDY

In paper II we have used a population based cohort including all 1089 singleton stillbirths in Stockholm during a 12 year period. Data was extracted from a web-based database including all stillbirths from gestational age ≥ 22+0 since 1998 in major Stockholm area. There is basically no risk for inclusion bias since all hospitals with delivery wards are represented in the Stockholm stillbirth group that is responsible for including cases in the database. Our study is the largest population study to date

comparing causes of stillbirth at different gestational ages in singleton pregnancies. In Stockholm, all parents of stillbirth are offered a standardized evaluation according to a structured test protocol evaluated in an earlier study [15], but the fact that the evaluation was not complete in all cases might have resulted in missed diagnosis in some cases. Particularly regarding autopsy is this a potential problem, since parents sometimes refused to consent to an autopsy, which might have led to missed diagnoses hereby influencing our results.
Autopsy and histopathological examination of the placenta are important since they have been shown to provide the most important diagnostic information [15, 26, 217]. In an Australian cohort study by Gordon et al. [172] histopathological examination of the placenta was performed in 952 (87%) of 1264 stillbirths. In our cohort, histopathological examination of the placenta was performed in 95.1% of the cases and autopsy was performed in 71.4% of the cases; these frequencies are high compared to previous studies that have not only shown lower frequencies but also a declining autopsy rate [218-220].

A problem when comparing our findings regarding cause of death with other studies is that few other relevant studies have been published. Moreover, previous reports use inclusion criteria different from ours. An American cohort study [22] from 2011, including 512 stillbirths, only included cases where a complete investigation had been performed. In a Dutch cohort study [24] from 2009, analyzing 750 stillbirth cases considering cause of death in different gestational ages, the authors comment that one reason for not including cases was “the doctor’s reluctance to include women with intrauterine fetal death because of an already known cause of fetal death at birth”. The authors presumed that this probably resulted in an underestimation of death especially due to placental abruption, known chromosomal abnormalities and major congenital anomalies. Our approach of including all singleton pregnancies with intrauterine fetal death in the study must also be considered strength of the study.

Another general limitation in the field is that studies addressing causes of stillbirth have used different classifications, making the comparison between them and i.e. our findings difficult. At the same time this further illustrates the need for an internationally accepted and applied classification system.

The cause of death was in paper II set by using the Stockholm classification of stillbirth, presented in paper I. The classification has been developed during audit work which can be considered a strength, since it is primarily aimed toward audit groups. It includes stricter definitions for cause of death, and the causes of death are subdivided in definite, probable, and possible, and allows for primary and associated causes of death. The definitions of cause of death, and the definitions of probability level, can be considered as subjective, which is a limitation of the classification. On the other hand, not including definitions of causes of death probably leads to a higher degree of subjectivity. The percentage of unknown/unexplained cases using the classification on the total cohort of stillbirths (multiple pregnancies included) was low (12.6%) and the

classification performed well (substantial) considering agreement of primary cause of death, which is a strength for both paper I and II.

Paper III and IV are both case-control studies that always have risks of bias, for example recall and observer bias. Since the cases in those studies were included after the stillbirth was identified, and most of the information was collected from the maternity medical records, we do not think that recall bias is an important problem in those studies, even though the risk cannot be ignored. However, such a problem might be pertinent to the preterm references in paper III, where the information was only received from the delivery record, since we did not have access to the maternity record. This also led to lack of information for the references considering BMI and smoking (at least if the patient smoked in the beginning of pregnancy and in week 32) as delivery records do not include this information. Anyway, smoking habits and BMI of the mothers were not included in the aims of this thesis.

The pathologist was not blinded for the groups in paper III and IV, implying a risk for observer bias. To decrease that risk a structured protocol for placental examination was used. By using a graded scale for CAM the risk for inter- and intra-observer variability can be further reduced [193] and we could obviously totally exclude the risk for inter-observer variability. A disadvantage of this approach is that the reproducibility of placental findings is not validated. On the other hand, analyzing variability in pathology reports was not within the scope of the present study.

Not including post-terms in the control group in paper III can be seen as a disadvantage of the study. However, post term pregnancies comprised only a minor part of the cases (n=11) thus the impact on our findings is probably not of great significance.

Paper IV is to our knowledge the first study that exclusively uses preterm infants as both cases and controls (references) when studying infection/inflammation of placenta. We think this is strength of the study. A healthy control group of preterm liveborn infants is of course impossible to recruit. The exclusion of the liveborn infants, not showing sufficient viability to be transferred to neonatal intensive care unit, might be seen as a weakness in paper IV, since those very sick infants from the liveborn group could be of interest to study. However, this decision was made to get a more distinctive border between cases and references.

Not including supplementary molecular techniques for detecting bacteria in the blood from stillborn heart is an obvious disadvantage of the study method, inherent to our current routine examination protocol; even more so in the light of previous studies which have questioned the efficacy of cultures alone for detecting bacteria, suggesting the need for supplementary molecular techniques (i.e., PCR [88-90, 170]). This is an issue that needs to be considered and addressed in any future revision of our stillbirth examination protocol.

6 CONCLUSIONS

The Stockholm classification of stillbirth, presented in this thesis, is a classification system exclusively for stillbirths, consisting of 17 groups of causes of death and allowing for one primary and several associated causes if needed. The causes of death are graded in probability levels: definite, probable or possible. The classification is primary meant to be used by audit groups and it has showed low percentage of unclassifiable cases. When validating the classification the overall agreement for all investigators towards consensus was good.

When analyzing causes of stillbirth in different gestational ages in a 12 year cohort, including all singleton stillbirths with gestational age ≥ 22+0 in major Stockholm area there was an overrepresentation of placental abruption and preeclampsia/hypertension in premature stillbirth, and umbilical cord complications and infection in term/post-term stillbirth. There was also a higher proportion of infection or histological CAM in post-term stillbirth compared to term stillbirths. The findings might have important implications for re-evaluating current praxis concerning time for induction of labor in post-term pregnancy.

In our case control study of placentas from term stillborn cases and liveborn controls histological CAM (especially severe: grade 2-3), villous immaturity, chronic villitis, SGA and maternal overweight, but not FIR, were independently associated with risk for stillbirth at term. These results indicate that the presence of CAM, especially severe, is a risk factor for fetal death in term pregnancies whereas FIR is not.

In our case control study of placentas from early preterm (gestational week 22-32) stillbirths and live births we found SGA and histological “CAM without FIR”, but not “CAM with FIR,” to be associated with a higher risk for stillbirth in early preterm pregnancies. These results indicate that the presence of CAM is not a risk factor for fetal death if FIR exists, but it is a threat to the fetus if FIR does not occur. Further research is needed to clarify if the development of FIR is actually protecting the fetus from death, a finding that of course could be of great importance for the understanding of mechanisms of stillbirth.

7 FUTURE RESEARCH

This thesis gives rise to a number of new questions that could be addressed in future research. I will here express some thoughts about new studies that can be continuations of the thesis work.

None of the six “21 century classifications” is approved as an international consensus classification. When working with paper II, trying to compare our results with the few other publications that had compared stillbirths in different gestational ages, the need for an internationally approved classification became obvious, since all reports had used different classifications. An international classification should primary be designed for research use and include the goals mentioned earlier in Background and Discussion sections. In the process of developing a new classification it is necessary to include researchers that have developed and worked with the six “21 century classifications”, but it is equally important to involve other experts clinically active in the field of stillbirth.

The difference found in paper II regarding causes of death in different gestational ages, especially the high proportion of infection in post-term stillbirths but also the higher proportion of umbilical cord complications in term/post-term stillbirths, could be clinically significant and warrants further studies. Relations between primary and associated causes and the connection between risk factors and causes of death are also of great interest and should be investigated further.

Placenta is a most interesting organ with short life span and vital function for the fetus during pregnancy. In paper III and IV we have focused on histological CAM and FIR, both generally believed to represent the findings in placenta due to an ascending microbial infection, mainly bacterial. Histological CAM has been clearly associated to stillbirth and was so also in our studies. Other studies have also raised the question if histological CAM and FIR are connected to other immunological processes; especially FIR has been associated with spontaneous start of labor. Our results showing that (severe) histological CAM but not FIR was independently associated with risk for stillbirth at term are intriguing and warrant further investigations into the links between placental inflammation, infection and stillbirth. For example we need to use supplementary molecular techniques to identify specific bacteria that may be involved in the histological CAM in stillbirths, and analyze whether they are different from bacteria involved in the histological CAM in live births. There is some evidence that specific bacteria as Ureaplasma or Mycoplasma might be involved more often in stillbirths. Further studies are also needed to elucidate whether FIR in term pregnancies is unrelated to the mechanisms of stillbirth or even confer some kind of protective action.

In our study of early preterm (gestational week 22-32) stillbirths and live births we found histological “CAM without FIR”, but not “CAM with FIR,” to be associated with a higher risk for stillbirth. We also found that FIR mostly occurred together with

severe CAM, indicating that FIR does not appear until CAM gets severe. Further research is needed to elucidate if the development of FIR is actually protecting the fetus from death, a finding that of course could be of great importance in the understanding of stillbirth.

Chronic villitis, basically a manifestation in late pregnancy, is clearly less frequent than CAM at term and much less well-studied in the context of stillbirth. Our results clearly demonstrate that chronic villitis is a risk factor for stillbirth at term; further studies are needed to elucidate the paucity of chronic villitis in premature placentas as well as the exact underlying pathogenetic mechanism contributing to fetal death, i.e. immunological- or virus-related.

8 SVENSK SAMMANFATTNING

Bakgrund: Den perinatala dödligheten (fosterdöd samt barn som dör innan sju dagars ålder) har minskat i Sverige de senaste decennierna. Fr.a. har tidig neonatal död (barn som dör innan sju dagars ålder) och intrapartal död (död under förlossning) minskat. Antenatal död (död innan förlossningsstart) har inte minskat i samma grad och för att kunna minska den krävs ökad kunskap om orsakerna till intrauterin fosterdöd (IUFD). Undersökning av placenta (moderkakan) och obduktion av fostret samt perinatal audit minskar andelen oförklarliga fall. I Stockholm bedöms samtliga IUFD-fall i audit efter utredning enligt ett utförligt provtagningsprotokoll, där bl.a. placentaundersökning ingår. Klassifikationssystem underlättar bedömningen av dödsorsak vid IUFD. Det finns > 35 olika klassifikationssystem för perinatal död men ingen konsensus kring vilket som är att föredra. Få studier har tittat på dödsorsaker relaterade till gestationsålder även om flera studier har analyserat enskilda dödsorsaker separat. Flera studier har visat att infektion är en vanlig orsak till IUFD, fr.a. i prematura graviditeter. Olika smittämnen kan orsaka olika former av inflammation i placenta. Bakterier anses orsaka ett maternellt inflammationssvar i form av histologisk akut chorioamnionit (CAM: inflammation av fosterhinnor) och härefter ett histologiskt fetalt inflammationssvar (FIR) i form av vaskulit (inflammation i kärl i placenta och/eller navelsträng) samt funisit (inflammation i navelsträngens stroma), medan virus anses orsaka kronisk villit (kronisk inflammation i placentaparenkymet).

Metod: I arbete I utarbetades ett klassifikationssystem för IUFD, ”the Stockholm classification of stillbirth”. Klassifikationen validerades gällande överensstämmelse i diagnossättning mellan användarna. I arbete II jämfördes, enligt klassifikationen, primära samt primära + associerade dödsorsaker i en kohort inkluderande samtliga 1089 IUFD-fall från enkelbörds graviditeter i Stockholmsområdet under 12 år. Fallen delades in i extremt prematura (gv. 22+0 till 28+6), måttligt prematura (gv. 29+0 till 36+6), fullgångna (gv. 37+0 till 40+6) och överburna(gv. ≥ 41+0).

Arbete III och IV utgörs av två fall-kontrollstudier där enkelbördsplacentor insamlats från; 126 fullgångna IUFD-fall och 273 fullgångna levande kontroller (arbete III) samt 112 prematura IUFD-fall (gv. 22+0-32+6) och 166 levande födda prematura referenser i matchade gv. (arb IV). Samtliga placentor undersöktes av en erfaren perinatalpatolog enligt ett standardiserat protokoll. Fokus var CAM och FIR (arbete III-IV) samt kronisk villit (arbete III).

Resultat: **Arbete I:** Utarbetande och validering av en klassifikation av dödsorsaker vid IUFD, ”the Stockholm classification of stillbirth”, vilken består av 17 dödsorsaksgrupper och tillåter fler än en dödsorsak. Dödsorsakerna har definierade kriterier och flera av dem är uppdelade i säkert, troligt och möjligt orsakssamband med IUFD. Validering visade god överensstämmelse mellan användarna (Kappa 0.70). I 89% av fallen var minst 4/6 av användarna överens om primär dödsorsak.

Arbete II: De prematura fallen hade oftare placentaavlossning och preeklampsi/hypertoni medan de fullgångna/överburna fallen oftare hade infektion och navelsträngskomplikation. Infektion var också vanligare i den överburna gruppen jämfört med den fullgångna gruppen och i den extremt prematura gruppen jämfört med den måttligt prematura gruppen.

Arbete III: IUFD-gruppens mödrar var äldre, hade högre BMI och hade oftare haft missfall. Barnen i IUFD-gruppen var oftare ”small for gestational age” (SGA) och deras placentor hade oftare en låg vikt i förhållande till graviditetslängden. CAM var vanligare hos IUFD-fallen (65.1% vs 40.7%) men då CAM delades i lätt respektive allvarlig CAM sågs endast skillnad avseende allvarlig CAM, som var vanligare hos IUFD-fallen (33.3% vs 11.4%). Allmän bakterieodling av IUFD-fallens hjärtblod kunde inte verifiera en bakteriell orsak till CAM eller FIR.
Kronisk villit var vanligare hos IUFD-fallen (18.3% vs 5.1%). Vaskulit och/eller funisit, trombos, omogna placentavilli samt infarkt var vanligare i IUFD-placentor. Multivariat logistisk regressionsanalys visade att CAM (speciellt allvarlig CAM) omognad av villi, kronisk villit, SGA och BMI ≥ 25 var förenat med ökad risk för IUFD. Vaskulit och/eller funisit föll inte ut som en oberoende riskfaktor för IUFD.
Arbete IV: Kronisk eller graviditetsinducerad hypertoni var vanligare hos mödrarna i fallgruppen medan preeklampsi var vanligare hos mödrarna i referensgruppen. Det var vanligare med SGA i fallgruppen och deras placentor hade oftare en låg vikt i förhållande till graviditetslängden medan referensplacentorna oftare hade accelererad villusmognad. Ingen skillnad sågs gällande kronisk villit. Det var ingen påvisbar skillnad mellan fall och referenser gällande CAM (33% i båda grupperna), inte heller om man graderade CAM i grad 1-3. FIR var vanligare i referensplacentorna (25.9% vs 14.3%). Alla placentor med FIR (utom ett fall) hade CAM. Placentor med CAM delades in i ”CAM med FIR” och ”CAM utan FIR”. ”CAM med FIR” var vanligare i referensplacentorna (25.9% vs 13.4%) medan ”CAM utan FIR” förekom oftare i IUFD-placentorna (19.6% vs 7.2%). Allmän bakterieodling av IUFD-fallens hjärtblod kunde inte verifiera en bakteriell orsak till CAM eller FIR.
I den multivariata logistiska regressionsanalysen var SGA och ”CAM utan FIR” oberoende riskfaktorer för IUFD men inte ”CAM med FIR” eller kronisk villit.

Betydelse: “The Stockholm classification of stillbirth”, har visat sig ge en hög grad av klassificerbara fall samt god samstämmighet mellan användare. De definierade kriterierna samt graderingen av sannolikhetsgrad för flertalet dödsorsaker gör att fall som har för få eller svaga fynd i utredningar för en säker dödsorsak ändå kan få en diagnos. Kunskap om hur dödsorsaker bland IUFD-fall fördelar sig i olika gestationsåldrar kan vara kliniskt viktig och användas för att utveckla preventionsstrategier som skärpt/riktad övervakning av vissa gravida. I en kohort av IUFD-fall från enkelbördsgraviditeter under 12 år i Stockholm var det mest överraskande fyndet den höga andelen infektioner/histologisk CAM i graviditeter ≥41veckor, vilket kan få konsekvens för vid vilken tidpunkt man ska överväga induktion av förlossning.
Det gick varken i arbete III eller arbete IV att verifiera en bakteriell orsak till CAM eller FIR i odlingar från IUFD-fallens hjärtblod. Detta kan bero på att endast allmän odling utfördes. Studier har visat att mer avancerad teknik identifierar bakterier som t.ex. Ureaplasma och Mycoplasma, vilka kan vara svåra att identifiera med traditionell odlingsteknik. Svårigheten att verifiera en bakteriell orsak kan också bero på att CAM och FIR inte orsakas av infektion. Dock tyder fynden i de två fall-kontroll studierna på att CAM (fr.a. allvarlig CAM), men inte FIR, är en riskfaktor för IUFD i fullgången

graviditet. I tidig prematur graviditet är CAM en riskfaktor för IUFD om inte FIR uppstår men resultaten antyder att FIR skulle kunna ge ett visst skydd mot IUFD i tidig prematur graviditet. Ytterligare studier behövs för att klargöra om utveckling av FIR faktiskt skyddar fostret från IUFD, en konsekvens som naturligtvis skulle kunna vara av stor betydelse för förståelsen av infektions-/inflammationsmekanismer vid IUFD. Kronisk villit var associerat med en ökad risk för IUFD hos de fullgångna men inte hos de prematura fallen. Bakgrunden till detta är oklar, möjligen är det prematura fostrets immunsvar för omoget för att trigga igång ett immunsvar i form av kronisk villit vid t.ex. en virus infektion.

Slutsats: Med hjälp av de fyra delarbetena har vi; arbetat fram en klassifikation som har låg inter-observationsvariabilitet och låg andel oförklarade fall och är tänkt att användas av audit-grupper som arbetar med IUFD; fått fördjupad kunskap om placentapatologi vid IUFD, fr.a. gällande inflammation/infektion i fullgången respektive tidig prematur graviditet; fått fördjupad kunskap om dödsorsaker i olika graviditetslängder.
Vi hoppas att arbetena ska bidra till ytterligare kunskap om fr.a. inflammatoriska processer vid IUFD och att skillnaden avseende dödsorsaker i olika graviditetslängder kan leda till en mer adekvat riskbedömning i olika graviditetslängder. Vi hoppas också att fler föräldrapar ska få en möjlig förklaring till varför IUFD inträffat.

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