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LJUNGAN VIRUS-

A NOVEL PATHOGEN

EFFECTS ON THE PREGNANT FEMALE AND HER OFFSPRING

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"Then the king of Egypt spoke to the Hebrew midwives, one of whom was named Shiphrah and the other was named Puah; and he said, "When you are helping the Hebrew women to give birth and see them upon the birth stool, if it is a son, then you shall put him to death; but if it is a daughter, then she shall live." But the midwives feared God, and did not do as the king of Egypt had commanded them, but let the boys live. So the king of Egypt called for the midwives and said to them, "Why have you done this thing, and let the boys live?" The midwives said to Pharaoh, "Because the Hebrew women are not as the Egyptian women; for they are vigorous and give birth before the midwife can get to them." So God was good to the midwives, and the people multiplied, and became very mighty. Because the midwives feared God, He established households for them. Then Pharaoh commanded all his people, saying, "Every son who is born you are to cast into the Nile, and every daughter you are to keep alive."

Exodus 1:15

I dedicate this work to my parents:
Anne-Renée and Olof

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3. Niklasson B, Samsioe A, Papadogiannakis N, Kawecki A, Hörnfeldt B, Saade G and Klitz W. Association of Zoonotic Ljungan Virus with Intrauterine Fetal Deaths. *Birth Defects Res A Clin Mol Teratol.* 2007 Jun;79(6):488-93

4. Kallies R, Niklasson B, Samsioe A, Hörnfeldt B, Mantke OD and Niedrig M. Ljungan Virus RNA Persists in Infected Laboratory Mice. *Manuscript submitted.*

5. Samsioe A, Sjöholm Å, Niklasson B and Klitz W. Fetal Death Persists through Recurrent Pregnancies in Mice Following Ljungan Virus Infection. *Birth Defects Res B Dev Reprod Toxicol.* *In press.*

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Thesis at a glance

I. Prenatal Viral Exposure Followed by Adult Stress Produces Glucose Intolerance in a Mouse Model.

It has been suggested that the intrauterine environment may influence metabolic diseases, especially diabetes mellitus, occurring in adult life and that adult stress may promote disease outcome. Using a mouse model, we tested whether *in utero* exposure to Ljungan virus (LV) followed by adult exposure to stress could induce diabetes. The influence of duration, in pregnancy, for viral exposure was also assessed. Pregnant CD-1 mice were exposed intraperitoneally (i.p.) to LV or normal saline on gestational days 4, 8, 12, and 17. Adult male mice from these pregnancies were stressed by being kept in shared cages. Outcome variables were: body weight, epididymal fat weight, baseline glucose, glucose tolerance test (blood glucose at 60 and 120 min.) and serum insulin levels.

We found that only male mice that had been both infected *in utero* and stressed developed obesity, increased epididymal fat, decreased glucose tolerance, increased serum insulin levels and overt developed diabetes mellitus. The diabetic state at the age of 11 weeks was more severe in mice whose mothers were infected earlier compared to later in pregnancy.

This work demonstrates that a type-2 diabetes-like disease can be induced by intrauterine LV infection in combination with postnatal stress in a mouse model. This may suggest that early *in utero* viral insult with LV in combination with stress can be of significance for the development of diabetes mellitus in humans.

II. Intrauterine Death, Fetal Malformation, and Delayed Pregnancy in Ljungan Virus–Infected Mice.

Epidemiological, clinical and zoological evidence suggests a possible causal role for LV in intrauterine fetal death (IUFD) in humans. In order to further investigate the possible role of LV in fetal and reproductive pathology, we studied the occurrence of IUFD, fetal malformations, neonatal death and delayed pregnancy in laboratory mice infected with LV under controlled conditions.

In three experiments, a total number of 28 CD-1 female mice were infected with LV two days after conception. Of these, 18 were in addition, subjected to stress, *i.e.* having their body weight measured and subjected to glucose tolerance test, while 10 were not. LV infection in combination with stress resulted in a high frequency (>50 %) of IUFD, malformations and neonatal death among pregnant animals. In the groups of non-infected animals subjected to stress, and infected animals not subjected to stress, only occasional cases of the aforementioned complications occurred. A delay in time to first pregnancy and births was also observed in pairs infected *in utero*.

Having established a possible role for LV for the development of fetal and neonatal pathology, especially in combination with stress and given the fact that LV is prevalent in different species of wild rodents in both Europe and the U.S.A. These findings suggest that LV infection can be an important factor not only in wild rodent population dynamics but also strengthen a possible LV-related pathogenic role for humans in similar clinical contexts.

III. Association of Zoonotic Ljungan Virus with Human Intrauterine Fetal Deaths.

This study aimed to investigate whether LV may be a zoonotic pathogen in humans. Population fluctuations of native rodents (the natural LV reservoir) in the north of Sweden were compared to the incidence of IUFD using the Swedish national hospitalization data base. Furthermore, formalin-fixed tissues from cases of IUFD where no etiology had been established were examined using a LV-specific monoclonal antibody. As controls placentas (*n*=20) from normal pregnancies occurring during the same time period and in the same area as the IUFD cases were used. We found that the variation in the incidence of IUFD closely tracked the fluctuations in the size of native rodent populations. In addition, LV was detected in brain tissue and placenta by immunohistochemistry (IHC) in 40 % (4/10) and 50 % (5/10) of IUFD cases, respectively. In none of the 20 control placentas from normal pregnancies was LV detected. These data raise a firm suspicion that LV may be of major clinical importance regarding IUFD in humans, at least in parts of the world where LV-infected rodents are prevalent.

IV. Ljungan Virus RNA Persists in Infected Laboratory Mice.

In this Paper, using quantitative real-time RT-PCR for the detection of LV RNA, we investigated whether persistent infection could be established in mice and the temporal dynamics of viral load in various organs was characterized. In both the acute and chronic phase of infection, LV was found in all organs. In the acute phase, the highest viral load was found in the brain ($>10^{10}$ gene equivalent numbers/g tissue), which was more than 10 times higher compared to that of the secondarily most severely infected organ (the pancreas) and more than 100 times more than that of the heart, lungs and intestine. In addition, we found histopathological evidence of encephalitis that corresponded to clinical signs of disease, *i.e.* uncontrolled movements. Thirty percent of animals with encephalitis died. The viral load decreased during the time but was still detectable in all organs after 174 days. The Ljungan viral load in chronically infected animals was ~ 10^6 gene equivalent numbers/g tissue with no significant differences between organs. Furthermore, we also found evidence of persistent infection with LV in wild bank voles (*n*=9/13) with viral load in various organs similar to what was found in mice during the chronic phase of infection. In conclusion, we demonstrate that LV RNA persists in infected laboratory mice and cause a systemic infection that in the acute phase shows a preference for brain tissue and both clinical and histopathological evidence of disease, *i.e.* encephalitis and death. The evidence of persistence of infection is an important factor when addressing the possible pathogenic role of LV in humans.

V. Fetal Death Persists through Recurrent Pregnancies in Mice Following Ljungan Virus Infection

This study explores the possible role for persistent LV infection in sequential offspring to female mice initially infected with LV. Following i.p. infection on gestational day two, female mice (*n*=15) and their female offspring (*n*=20) were monitored regarding occurrence and frequency of perinatal deaths. Dams were followed for two or more consecutive pregnancies, three mating attempts and compared to non-infected mice (*n*=15), both the infected mice and their offspring showed significantly higher frequency of perinatal death in their pregnancies. No differences between the primarily infected animals and their offspring regarding these complications were found. These findings, *i.e.* the occurrence and similar frequencies of perinatal deaths in LV infected female mice and their offspring, suggest that LV may establish itself as a chronic infection that further persists and impacts through vertical transmission of the virus.

VI. Ljungan Virus Present in Human Intrauterine Fetal Deaths Diagnosed by Both Immunohistochemistry and PCR

Following leads from animal studies, we aimed to examine the presence of LV in cases of human IUFD. A prospective study was performed on five cases. Frozen and formalin-fixed specimens from IUFD cases (brain, lung, liver, spleen and placenta) were collected from the Stockholm area (central Sweden), plus three formalin-fixed brain tissues from Boston, U.S.A., also stillbirth cases. Presence of LV antigen was investigated using IHC and the presence of LV RNA was detected by real-time RT-PCR. Formalin-fixed brain samples from 18 induced abortions (gestational week 15-22) were used as controls. These were terminated pregnancies because of the diagnosis Trisomy 21.

The combined results from the IHC and real-time RT-PCR assay on all five IUFD cases demonstrated presence of LV in at least one organ with either diagnostic tool. One case was positive in the brain with both techniques. One of the three brain samples from the U.S.A. was positive with IHC and one of the control specimens was also IHC positive.

Degradation of tissue and the low copy number of viral RNA may limit the possibility to detect LV in tissues using IHC and RT-PCR.

These findings call for further studies to investigate the implication of LV as a pathogen in stillbirths.

List of abbreviations

AR	Antigen retrieval
BB-DP	BioBreeding diabetes-prone rat
BMI	Body mass index, kg/m²
BW	Body weight
CNS	Central nervous system
CMV	Cytomegalovirus
CPE	Cytopathogenic effect
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis viruses
FHR	Fetal heart rate
GAD	Glutamic acid decarboxylase
GBS	Guillain-Barré syndrome
GD	Gestational day
GDM	Gestational diabetes mellitus
GK	Goto-Kakizaki rat
GMK	African green monkey kidney
GST	Glutathione-S-transferase
GTT	Glucose tolerance test
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
i.c.	Intracerebrally
ICD	International classification of disease code
IFA	Indirect immunofluorescence assay
IHC	Immunohistochemistry
IUFD	Intrauterine fetal death
IUGR	Intrauterine growth restriction
i.p.	Intraperitoneally
JEV	Japanese encephalitis virus
LADA	Latent autoimmune diabetes in adults
LCMV	Lymphocytic chorionmeningitis virus

LGA	Large for gestational age
LV	Ljungan virus
MAb	Monoclonal antibody
MBR	Medical birth register
MHV	Mouse hepatitis virus
NE	Nephropathia epidemica
NOD	Non-obese diabetic mouse
PIN	Personal identity number
p.i.	Post infection
PNM	Postnatal mortality
RSA	Recurrent spontaneous abortion
RT-PCR	Reverse-transcriptase polymerase chain reaction
SCB	Statistiska centralbyrån, Statistics Sweden
SIDS	Sudden infant death syndrome

Summary

Ljungan virus (LV) was isolated in the mid 1990s from a native rodent, the bank vole *(Myodes glareolus*), trapped in the Ljungan valley of central Sweden. It is a member of the parecho virus genus in the picorna virus family. A consistent characteristic feature of the LV is that it is difficult to cultivate in tissue culture and therefore difficult to isolate and diagnose. The virus grows to only low titers in both tissue culture and organs infected under controlled conditions. These features in combination with a poor humeral immune response constitute a diagnostic challenge.

Interest in LV heightened when it was found that the virus is associated with diabetes, neurological diseases and reproductive disorders in its natural reservoirs – small wild rodents.

This research explores the influence of LV infection in laboratory mice through several experimental approaches. Male mice infected during the first postnatal days were persistently infected during the entire five month (174 days) long follow-up period, as determined by real time RT-PCR (Paper IV). When dams were infected early in pregnancy, the litter experienced a high frequency of perinatal death. In addition, malformations were observed in some of the offspring (Paper II). Females infected intraperitoneally, and females infected *in utero*, were followed over time with the problems remaining through three consecutive pregnancies (Paper V). In addition, it was observed that the offspring of infected mice had delayed reproduction. This phenomenon would, in wild rodents, have major consequences for their population dynamics (Paper II). Another effect produced by LV infection was that surviving offspring of infected females developed diabetes at 10-15 weeks of age (Paper I). These animals had intact β-cells, increased abdominal fat, hyperinsulinemia and impaired glucose tolerance, suggestive of a type-2 diabetes-like state. The earlier in pregnancy the infection took place, the more severe the diabetes in the mature offspring. Mild stress was necessary for LV to induce the diseases. Infection or stress alone did not cause any disease, but animals subjected to the combination of stress and LV infection developed the reproductive disorders and diabetes (Paper I and II).

The association between LV and disease in the wild reservoirs, and the fact that these diseases can be reproduced in a mouse model under controlled conditions, engendered interest in the possibility of whether the virus might be responsible for these same conditions in humans. An earlier epidemiological study had seen associations between human type-1 diabetes, Guillain-Barré syndrome, lethal myocarditis and the abundance of the animal reservoir of LV. Our follow-up study then found a striking association between IUFD in humans and the abundance of wild rodents. This finding led to an investigation of the presence of LV in human IUFD cases. In a small pilot study, LV was found in approximately half of the cases investigated using immunohistochemistry (Paper III).These findings were later confirmed by a recently developed real-time RT-PCR assay (Paper VI).

This work probes entirely new ground on a novel infectious agent. The first animal model of pathogenesis from this agent has been published. While the excitement of the results must be tempered somewhat due to the ongoing development of new diagnostic methods, the potential importance of the pathogen is already evident.

1. Introduction and background

1:1 Zoonotic viruses causing acute and chronic diseases

A zoonosis is an infectious disease that is transmitted between animals and humans. Animals can act as both reservoirs and vectors, spreading viral diseases to other animals and/or humans. The feasibility to identify and associate zonootic viruses with human disease is influenced by its biological characteristics. Viruses that are difficult to detect and/or cultivate pose diagnostic hurdles in the establishment of a causal relationship between virus and disease. Zoonotic viruses, which are under low selective pressure for efficient growth in a human host, may result in low pathogen concentration and thus make viral identification difficult. In some zoonotic diseases, the human is considered a "dead end", meaning that the infectious agent is not spread further. In a dead end host, the concentration of the infectious agent is often low. This fact may not have a major effect on disease outcome, but low pathogen concentration often decreases the prospect of identification. Incubation time, incidence and severity of disease are factors that all influence the difficulty in establishing a link between a potential pathogen and human disease. Epidemiological observations of a vector, mainly regarding its population dynamics, often precede the development of diagnostic tools necessary for linking the agent to a disease[1,2].

However, it is an advantage for a pathogen that must be transmitted between individuals in order to maintain its existence to be able to grow to high titers in the host. A high concentration of a pathogen greatly increases its chance of transmission and also the possibility of diagnosing that particular agent.

Hanta virus is one example of a pathogen transmitted by rodents, infecting humans and following rodent density cycles. The Hanta viruses are a widely distributed group of viruses infecting various rodent species, which serve as both reservoir and vector.

Despite a short incubation time and a clinical picture which is both severe and well known from early in the last century[3], it took scientists several decades to identify the etiologic agent. In fact, it was only in 1978 and 1984 in Asia and Europe, respectively, that these viral agents were properly identified as human pathogens[4-8]. Puumala virus, causing Nephropathia epidemica (NE), was first identified in Finland[9,10], while Hantaan virus causing Hemorrhagic fever with renal syndrome was isolated in Korea[6,11-14]. The vector and natural reservoir of Puumala virus is a small rodent, the bank vole *(Myodes glareolus),* [Fig. 1] [5,10,15-19].

Fig. 1 *The Bank voles (Myodes glareolus).* *Foto: B. Niklasson*

The discovery of Puumala and Hantaan viruses illustrates the value of integrating epidemiology, vector biology and microbiology in order to identify the cause of a zoonotic disease[8,11,15,16].

While Hanta virus is classified in the family of *Bunyaviridae*, Ljungan virus (LV) belongs to the *Picornaviridae* family. The process of the discovery and identification of LV shows many similarities to that of Hanta viruses. Hanta and Ljungan are both zoonotic viruses, which use small rodents as their reservoirs. Additionally, both viruses are particularly difficult to cultivate, and primary isolation in tissue culture is therefore rarely successful. Epidemiological studies associating the reservoir with disease in humans and isolating the virus from the reservoir have been the starting point, both for research on Hanta virus in the past, as well as for current investigations of the possible role for LV as a human pathogen[8,9,15,16].

The distribution of the bank vole is widespread in most parts of Europe and Asia. It is probably one of the most prevalent small mammalian species in these regions, and it is the most common wild mammal in Sweden[17,20]. Small rodent populations in the southern-most parts of Sweden are non-cyclical. In contrast, populations in the north show distinct fluctuations in abundance on a three to four year cycle[19-21].

The boundary between the cyclic populations in the north of Sweden and the non-cyclic populations in the south corresponds to the "*Limes Norrlandicus"*, a bio-geographical border running from the west coast of Sweden to the east coast (Fig. 2). This border separates the northern boreal (or taiga) zone from the southern boreal-numeral zone[18,22]. During peak abundance, voles may be >300 times more common than immediately after population declines in northern Sweden[17-19]. Long-term records (>10 years) on small rodent abundance are, from a global perspective, scarce. In Sweden, however, such data is available as part of the ongoing National Environmental Monitoring Program run by The Swedish Environmental Protection Agency. However, this monitoring is mainly restricted to the northern, cyclic region, but records are available also from the southern regions which show no cyclic rodent density[20].

The discovery of Hanta virus in Asia and Europe was directed by the association between human cases and rodent contact as well as by epidemiological studies between rodent density and incidence of disease[23-26].

Fig. 2 *"Limes Norrlandicus", a bio-geographical border running from 59 °N on the west coast of Sweden to 61 °N on the east coast.*

The fact that many picorna viruses cause broad spectra of diseases in humans from neurological conditions to infections such as myocarditis was i.a. what initiated similar epidemiological investigations when LV was isolated in the mid 1990s[27].

In fact, type-1 diabetes, lethal myocarditis and Guillain-Barré syndrome (GBS), were found to be associated with rodent abundance in northern Sweden. Time series of rodent abundance in Sweden are shown below (Fig. 3) and the association between rodent density and lethal myocarditis and diabetes type-1 is shown in Fig. 5 and 6, respectively[28].

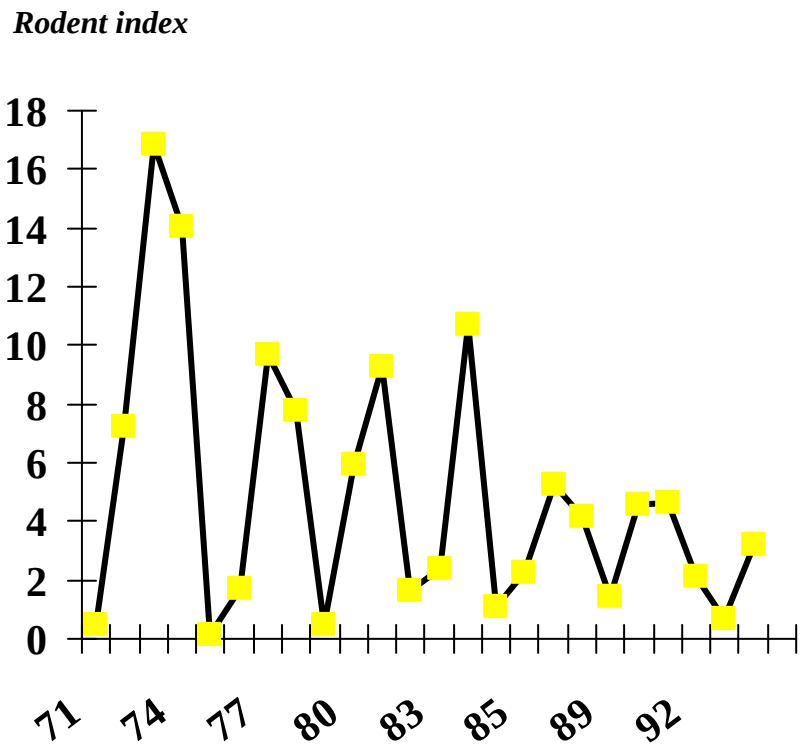

Fig. 3 *Rodent index. Data is from the National Environmental Monitoring Program by the Swedish Environmental Protection Agency.*

In brief, the procedure for monitoring these rodents is that of “snap-trapping”. It is performed in 60 one hectare ranges of measurement areas. On each trapping occasion, ~ 3,000 traps are set over three consecutive days, amounting to ~ 9,000 "trap-nights". Monitoring has been running every spring (end of May) and fall (end of September) since 1971[20,29].
(http://www.emg.umu.se/personal/lankar/hornfeldt/index3.html)

1:2 Intrauterine fetal death/stillbirth

Despite the magnitude of the problem of unexplained stillbirths, relatively little attention has been paid to fetal demise during the past few decades. Stillbirth and neonatal death are ten times as frequent as sudden infant death syndrome (SIDS) but have attracted significantly less scientific attention[30,31].

Stillbirth accounts for a large proportion of all perinatal losses, although the causes remain, to a large extent, ambiguous[30]. Most investigators attempt to classify stillbirth into diagnoses with or without known cause[32]. Rarely is the possibility to distinguished between cases with distinct etiology, *e.g.* from infection, trauma or genetic diseases and cases where fetal death is a consequence of other, mainly maternal disease, such as pre-eclampsia or maternal autoimmune diseases, *i.e.* systemic lupus erythematosus (SLE) or diabetes mellitus[33,34]. Most maternal diseases, mainly SLE, diabetes mellitus and pre-eclampsia which result in fetal death have no established etiology[33,35]. Intrauterine growth restriction (IUGR) is known to be associated with placental abruption. IUGR has frequently been present when the abruption occurred. Likewise, intrapartum asphyxia resulting in stillbirth often takes place when fetuses already are growth restricted[36]. Many conditions that have been suggested as explanations of IUFD/stillbirths, such as IUGR, previous preterm delivery, stillbirth recurrence, placental infarction, necrosis and vascular thrombosis or placental abruption and obesity are also examples of disorders with no universally accepted etiologies [37-41].

The differences in the incidence of unexplained stillbirths depend to a large extent on how the condition is defined. Definitions vary between countries, health organizations and investigators, as well as

between disease classification systems. These differences, in conjunction with the lack of an international consensus for the classification of perinatal loss in terms of birth weight and/or the length of gestation, make international comparisons difficult[35,42]. Another issue is that smallness for gestation has a demonstrable link with fetal death at the population level. Individually, however, each fetus may be either pathologically or physiologically small, and could be inappropriately classified if only weight for gestation is used [36,43,44].

Moreover, depending on gestational age, or age of the infant, death is classified into miscarriage, IUFD, or SIDS [35]. To illustrate the concern of differences in definitions, classification systems and the problems of international comparisons, two out of several classification systems are described [42]. There are more than 30 reported systems for classification of perinatal death[45,46]. A population-based cohort study was performed in the U.K. where two different classification systems were evaluated and compared, *viz.* the conventional Wigglesworth classification system and the ReCoDe (relevant condition at death)[44]. The same data processed regarding unexplained fetal death revealed that Wigglesworth left 66 % of cases unclassified, while using the ReCoDe system this decreased to only 15 % [36]. The specific major condition associated with fetal loss was severe IUGR, representing 43 % (Wigglesworth) or 58 % (ReCoDe) of the cases[47]. These circumstances do not become evident when perinatal mortality is presented in separate groupings for weight and gestational age. However, with new or traditional classification systems the etiology often remains elusive.

In the United States, the stillbirth rate has declined by approximately 17 % between 1985 and 2001 (from 7.8 to 6.5 deaths per 1,000 births), as compared to a 35 % decline in infant mortality over the same time period [35,48]. Around half of the stillbirths occur prior to 28 weeks of gestation and about 20 % are at or near term [30,49]. The incidence of IUFD from the 28th gestational week is currently reported to be 3 per 1,000 births in Sweden[50]. Neonatal mortality (live born infants who die within 28 days), accounts for 1.8 per 1,000 children born 2006 in Sweden[51].

A significant decrease in the incidence of stillbirth occurred after the introduction of Rh-immune prophylaxis. Since the introduction of *post-partum* rhesus prophylaxis in the 1970s, fetal morbidity and mortality have been reduced by ~90 percent[52-55]. Following the introduction of intrapartum monitoring has further decreased death during labor[31,56-58].

Intermittent electronic fetal heart rate monitoring (FHR) *vs*. intermittent auscultation has been evaluated. It was concluded that continuous monitoring did not show any apparent differences in the rates of low Apgar scores, need for resuscitation, or transfer to the special care nursery[59,60] unless done in risk deliveries *e.g.* SGA [61]. In addition, FHR monitoring was superior in detecting fetal acidosis. On the contrary, intermittent electronic FHR was associated with increased rates of surgical intervention and did not improve the outcome[62]. A small decreasing trend was notable for cerebral palsy in the group of children born preterm (prevalence 1.92 per 1,000 live-births). Only 5 % in this Swedish study of the prevalence had an obvious postnatal cause of cerebral palsy [63].

The immune system has to protect the pregnant female against infections, as well as tolerate the presence of the antigenically foreign placenta and fetus. In order to achieve this, it is proposed that regulatory T-cells, more specifically Th1 and Th2 cytokine balance, play an important role in protecting the pregnancy from rejection by the female. It has been speculated that pregnant mammals are at an increased risk of acquiring infectious diseases for which cell-mediated immunity is an important defense: however, the relevance of this in humans remains elusive[64-67].

Stillbirth has been associated with bacterial, protozoal and viral infections[68], and there is a broad variety of microorganisms that are capable of causing fetal infections. Three well known zoonoses in fetal medicine are toxoplasmosis (protozoa), listeriosis and brucellosis[69]. Among viral agents prominent are the human cytomegaly virus (CMV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), varicella, herpes zoster, rubella, parvovirus B19, measles and the hepatitis B and C viruses[70]. The *spirocheta pallida*, causing congenital syphilis which has adverse effects on pregnancy outcome, is also

a well known example of fetal pathogen. Syphilis is not only an issue in developing countries, since the disease is increasing in developed countries as well, and it is still a countable emerging fetal pathogen[71,72]. In Sweden, however, only one congenital case has been reported since 1997[73]. Bacterial infections are responsible for *in utero* acquired listeriosis, tuberculosis, and group B streptococcus infections[74]. Fungi, including *Candida albicans*, complete the circle of infectious pathogens. Infectious microorganisms may reach the fetus through the placenta (hematological) or may ascend through the birth canal. The quoted pathological agents threaten the health and life of the fetus directly by the biological derangements they cause and also by inducing abortion or premature birth. The clinical manifestations include retarded growth, central nervous system damage and skin lesions. The therapeutic measures vary, but, in general, are of limited value in cases of infections acquired *in utero.*

Infections may provoke stillbirth by a number of mechanisms, *e.g.* preterm labor and direct infection of the fetus, placental damage, chorioamnionitis or severe maternal illness[75-79]. Ascending infections with *E coli, group B streptococci* and *Ureaplasma urealyticum* are the most common bacterial causes of stillbirth[31]. In endemic areas, tropical diseases, mainly *Plasmodium falciparum* and *vivax*, are important causes of stillbirths[68,76,80-84]. In addition, genital infection with *Mycoplasma, Candida, Toxo-plasma gondii, Listeria monocytogenes, Leptospira, Treponema pallidum, Coxiella burnetii* and *Borrelia burgdorferii* have all been implicated as potential etiologic agents of stillbirth [33,35,58,68,74,76,85-90].

Parvo virus B 19 is also associated with stillbirth[68,76,91-104]. A Swedish study identified parvo virus B19 in 14 % of IUFD[99]. In contrast, less than 1 % of all stillbirths in the U.S.A. are associated with Parvo virus infection[76]. In addition, infection with Entero viruses has been shown to be associated with stillbirth[97,105,106]. Coxsackie viruses can cross the placenta barrier and induce villous necrosis, pancreatitis and *hydrops fetalis* [68,92,107]. CMV is one of the most common congenital infections among humans. While CMV has clearly been associated with placental damage leading to IUGR, its association with stillbirth remains controversial[68,70,76, 85, 108]. For infectious agents generally, both systemic and intrauterine infections can cause preterm labor with subsequent fetal risk[77]. Fetal bacteremia associated with ascending intrauterine infections was reported in 33% of pregnancies with positive amniotic fluid cultures *vs*. 4% with negative culture[109]. Genital mycoplasma species were detected in 23% of cultures from umbilical cords, suggesting that occult intrauterine infections are more common than traditionally recognized[109,110]. Delivery because of maternal or fetal infections accounts for 30%, preterm labor with rupture or intact membranes 25 % and spontaneous preterm labor the remaining 45 % of stillbirths[111]. Prematurity is associated with a 75 % risk of fetal death and a 50 % risk of long-term neurological disabilities[79,111-114]. Recurrent spontaneous abortion (RSA) is defined as three or more consecutive pregnancy losses prior to the 20th week of gestation. The etiology of RSA is often unclear and may be multifactorial, with much controversy regarding diagnosis and treatment. Reasonably accepted etiologic causes include genetics, anatomical, endocrine and placental anomalies, hormonal problems, infection, smoking and alcohol consumption, exposure to environmental factors, psychological trauma and stressful life events, and certain coagulation and immunoregulatory protein defects[115].

However, there remain large discrepancies between different classification systems, both in terms of percentage for risk factors as well as etiology. The proportion of stillbirth associated with infection varies between 10 and 25 % in developed countries[35]. Intrauterine infection has been implicated in several obstetric complications, *e.g.* IUGR (20-43 %), preterm birth (5-13 %) as well as perinatal death[116]. The precise role and the exact number of incidences remain unclear. The mechanisms behind perinatal viral infections vary depending on the specific pathogen. The infection always begins with maternal infection, following indirect placental infection, chorioamnionitis, funisitis and, with or without fetal infection. Some pathogens confer injury through more than one mechanism[117-121].

However, given the fact that over half of the stillbirths defy explanation, a great deal remains to be learned about this distressing outcome of many pregnancies[77,87,115, 122,123].

Definitions

Sweden

Living child = A newborn who is breathing or shows other evidence of life, such as heart beats, umbilical cord pulsations or definitive movements of voluntary muscles. This is irrespective of gestational or developmental age.

Stillborn or intrauterine fetal death, IUFD = A child deceased before or during labor and after 28 weeks of gestation or has a length of minimum 35 cm.

Perinatal mortality refers to the number of perinatal deaths per 1,000 births. The perinatal period begins with fetal viability and ends on the 7th day after delivery.

Perinatal death is the sum of stillbirths and early neonatal deaths [51].

International

Stillbirth refers to no signs of life, birth weight limit recommended to 500 gram, or gestational age of at least 28 completed weeks, or a body length of > 35 cm.

According to Office of Population Censuses and Surveys (OPCS) 1991, stillbirth rate is defined as:

$$\text{Stillbirth} = \frac{\text{Stillbirth} \times 1{,}000}{\text{Live birth} + \text{Stillbirth}}$$

Perinatal mortality or *perinatal death* refers to the death of a fetus or neonate (infant) and is the basis for calculating the perinatal mortality rate. Variations in the definition of perinatal mortality exist, specifically concerning the issue of inclusion or exclusion of early fetal and late neonatal death. Thus, The World Health Organization's definition, "Deaths occurring during late pregnancy (at 22 completed weeks of gestation or more), during childbirth and up to seven completed days of life", is not universally accepted. The perinatal mortality is the sum of the fetal mortality and the neonatal mortality.

Fetus refers to after the embryonic stage and before birth.

Fetal mortality refers to stillbirths or fetal death. It encompasses any death of a fetus after 20 weeks of gestation or >500 gram. In some definitions of *postnatal mortality* (PNM), early fetal mortality (week 20-27 gestation) is not included and the PNM may only include late fetal death and neonatal death. Fetal death can also be divided into death prior to labor, antenatal (*ante partum*) death, and death during labor, intranatal (*intra partum*, occurring during labor and delivery) death.

Early neonatal mortality refers to a death of a live-born fetus within the first seven days of life, whereas late neonatal mortality covers the time after 7 days until before 29 days. The sum of these two represents the neonatal mortality. Some definitions of PNM include only the early neonatal mortality.

The *perinatal mortality* rate refers to the number of perinatal deaths per 1,000 total births and is usually reported on an annual basis. It is a major marker to assess the quality of health care delivery. Comparisons between different mortality rates may be hampered by varying definitions, registration bias, and differences in the underlying risks of the populations. The perinatal mortality rate varies widely and may be below 10 for certain developed countries and more than 10 times higher in developing countries.

(WHO, Reproductive, Maternal and Child Health European Regional Office)

In the current International Classification of Disease codes (ICD), ICD-10 classification, stillbirth is included in the term perinatal mortality, which combines fetal death (after 22 completed weeks, or death of a fetus of > 500g) and newborn (death within seven days after birth).

Animal models for study of IUFD/stillbirth

Animal models have been used to study the pathogenesis of IUFD. Some models use infectious agents to address their role in inducing IUFD and other obstetric adverse outcome[123-128]. One example is Japanese encephalitis virus (JEV), where the mouse model shows findings indicative of persistence, latency and reactivation of the virus, in association with fetal death in the offspring[129,130].

Another model is Coxsackie B virus infection in the pregnant mouse, which results in severe generalized infection. Virus is detected in several organs, *e.g.* spleen, lung, liver, as well as blood and feces. Coxsackie B infection in the murine model leads to a high spontaneous abortion rate[131-134] and increases the incidence of stillbirth and *post partum* fatalities. It also has an effect on the future reproductive capacity of dams and increases the mortality rate of the offspring in consecutive pregnancies[133]. Other animal models include Brucella infection in mice, which repeatedly causes abortion[135], preterm birth and neurological disabilities[136]. Infectious and inflammatory models confirm the relationship between prenatal and postnatal or perinatal inflammation and the outcome of brain injury[128].

1:3 Diabetes mellitus

Diabetes mellitus (DM) is a group of chronic metabolic disorders characterized by hyperglycemia which results from defects in the secretion and/or effects of insulin. DM was first identified in the ancient world as a disease associated with “sweet urine" and excessive muscle wasting. Hyperglycemia leads to an excessive amount of glucose filtered into the primary-urine overriding the capacity for re-absorption of glucose in the proximal renal tubules, hence the term “sweet urine”. The hormone insulin is exclusively produced by β-cells in the pancreas. In patients with DM, the absence or insufficient production of insulin causes hyperglycemia. There are two major types of DM, type-1 and type-2.

Type-1 DM: In type-1 DM, the endocrine pancreas (islets of Langerhans) is the site for an inflammatory, autoimmune assault with destruction of insulin-producing β-cells leading to a decreased and eventually insufficient capacity for producing insulin. Autoantibodies toward β-cells are found in a majority of patients. This absolute lack of insulin is the hallmark of type-1 DM. The disease tends to occur in young lean individuals, usually before 30 years of age. Occasionally, elderly individuals may also present this form of DM. Of all patients with DM, approximately 10 % suffer from type-1 DM.

Type-2 DM: The pathophysiology of type-2 DM is fundamentally different from that of type-1 DM. It has two main features; insulin resistance and defect insulin production. Insufficient production of insulin or the inability of cells to respond to insulin leads to hyperglycemia and associated symptoms, *i.e.* polyuria, polydipsia, blurred vision and fatigue. This insulin resistance chiefly affects muscle and fat cells and is a major pathogenetic component in type-2 DM. Moreover, there is a relentless decline in β-cell function and number that further accelerates the diabetic process, *i.e.* increases the hyperglycemia.

Hales and Barker designed the hypothesis of “the thrifty phenotype”, referring to an offspring that is programmed to survive under conditions of poor nutrition[137]. This hypothesis is founded on the notion that the highest rates of neonatal mortality and the highest percentage of low birth weight in the U.K. during the 20th century were consistently found in economically deprived areas. British researchers postulated that impaired fetal and postnatal growth predispose to coronary heart disease, type-2 DM and the insulin resistance syndrome. Children, who were small at birth (“smallness and thinness”) and showed continuous slow growth during their first years, then accelerated quickly and finally, as adults, had a body mass index (BMI) above normal. These individuals suffered an increased risk of the aforementioned diseases. Since this original discovery, these observations have been reproduced in a

variety of studies and populations worldwide[138-141]. The survival benefit for the individual during periods of poor nutritional conditions is obviously of paramount importance but may, according to this hypothesis, create substantial risk for future morbidity, especially when nutrition becomes abundant[142]. Other factors (besides poor maternal nutrition) that may cause impaired fetal and infant growth may play a role in the pathogenesis of diseases, such as type-2 DM and the metabolic syndrome (a combination of obesity, cardiovascular disease and diabetes)[143,144]. In the Western World, women are usually well-nourished and, therefore, the intrauterine environment does not program for famine. Still, BMI and obesity increases, and so does the pattern of metabolic and functional diseases, such as type-2 DM, cardiovascular diseases, hypertension and insulin resistance[139,145,146]. It has also been suggested that pre-eclampsia may be associated with an increased risk of contracting of type-2 DM in the mother[144].

Gestational diabetes

Diabetes can occur temporarily during pregnancy, gestational diabetes mellitus (GDM), and is defined as glucose intolerance with onset during pregnancy. GDM adversely impacts the developing fetus, maternal health and pregnancy outcome[147]. It may also lead to increased neonatal morbidity and mortality[148], if left untreated. Approximately 3.5% of Swedish women will develop GDM. Risk factors for developing this condition include a previous diagnosis of GDM, age over 35 years and obesity. Twenty-five to fifty percent of women with GDM will eventually develop type-2 DM later in life, especially those who require insulin therapy pregnancy and those who remain overweight after delivery[147,149,150]. Prompt diagnosis of GDM is important since GDM carries increased risk for both mother and infant. For example, children of mothers with GDM may be large for gestational age (LGA), which poses a risk of trauma to both mother and baby during delivery such as maternal hemorrhage, infant asphyxia and shoulder dystocia concomitant to neonatal macrosomia (birth weight >4.500 g). The risk for the infant of hypoglycemia (plasma glucose <2.2 mmol/l), and hyperbilirubinemia (serum bilirubin ≥342 μmol/l) increases with rising plasma glucose levels by the pregnant women[151]. Infants born to mothers with GDM are also subject to a higher risk of severe respiratory problems after birth[152], and the risk of obesity and glucose intolerance in adulthood also is increased[141,149,153,154]. As previously mentioned, women with GDM are additionally at increased risk for developing overt type-2 or type-1 DM later in life[150], as well as pre-eclampsia and IUFD[155]. In addition, women with GDM and obesity run a significant higher risk of developing metabolic syndrome than women with only one of these conditions[147].Both diabetes type-1 and type-2 increases the risk of stillbirth[156,157]. The risk of Swedish women with type-1 DM is 4 to 5 times higher than among women without DM type-1[158]. The majority of IUFD in pregnant women with DM occurs in those with poor blood glucose control and complications such as macrosomia, polyhydroamnios, IUGR of the fetus and pre-eclampsia. Fetal death in uncomplicated pregnancy with GDM is rare[34].

Animal models for studies of diabetes mellitus

There are several established animal models for studying diabetes. These include type-1 diabetes-like models, such as the non-obese diabetic (NOD) mouse and the BioBreeding Diabetes-Prone (BB-DP) rat[132,159-164]. Several rodent models have also been established to study GDM[162,165]. To study type-2 DM[166], animal models like the Goto-Kakizaki (GK) rat are frequently used[167,168]. The lean GK rat is one of the best characterized rodent models for spontaneous type-2 DM[166,167]. These animal models have proven very valuable in improving our understanding of β-cell dysfunction and insulin resistance[169] during the unfolding of diabetes. To test the “the thrifty phenotype” hypothesis, animal studies have been performed in rats. Pregnant dams, fed a diet with reduced protein, and their offspring showed accelerated growth postnatally[143]. By being cross-fostered to normally fed lactating dams, and then weaned onto a normal diet, the offspring showed increased systolic blood pressure as compared to controls and some died prematurely[140].

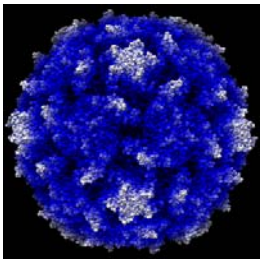

Fig. 4. *A picorna virion (Polio). These are the smallest RNA viruses, 20-30 nm in diameter. The icosahedral, non-enveloped virion contains polycistronic linear ss RNA of positive polarity that is polyadenylated at its 3'-end and has a small protein, VP_g attached to its 5'-end. The viral RNA is translated into polyprotein, which is processed into capsid-forming structural proteins and non-structural proteins important for viral replication.* *(Foto: N. Knowles)*

1:4 Ljungan virus

Ljungan virus was first isolated from a wild-trapped bank vole in the Ljungan valley, county of Medelpad, in the north of Sweden. The isolation of LV was made from feces and lung homogenate[27]. LV is part of the family *Picornaviridae,* which includes eight different genera: *Aphto, Cardio, Entero, Erbo, Hepato, Kobu, Parecho* and *Tescho* virus. LV belongs to the genus of *Parecho* virus, which is comprised of two species, human *Parecho* virus and LV. *Parecho* virus contains at least six distinct serotypes[170,171]. These were formerly known as human *Echo* virus types 22 and 23. LV is comprised of four known serotypes. The LV particles are 18-30 nm in diameter and consist of single stranded RNA genome of about 7,000 base pairs. LV: s "2A gene" is doubled, a feature that separates it from most other known *Picorna* viruses[172,173]. The physicochemical properties of LV make the virus particles very stable[174]. The transmission route(s) of the LV remain unknown, but many picorna viruses have a fecal-oral transmission route.

Recently, three more proposed genera, *Sapelo* virus, *Seneca* virus and *Tremo* virus have been added to the order. *Entero* virus*, Hepato* virus*, Kobu* virus*,* and *Parecho* virus infect humans, while *Aphto* virus*, Erbo* virus, *Tescho* virus and *Cardio* virus are animal pathogens. The genus *Cardio* virus is divided into two species: Theiler viruses (TMEV) and the Encephalomyocarditis viruses (EMCV)[175,176]. Although rats and mice are the natural hosts for EMCV, these cardio viruses have been found to infect many animal species, including pigs, rodents, elephants and macaques[177-179]. There have been suggestions that humans can be infected with cardio virus, however, its effect as a human pathogen remains controversial[180-183].

Ljungan virus and disease

Zoological data, collected in Sweden as early as in the 1930s, was able to link the rodent population density to the presence of Nephropathia epidemica (NE)[3]. The first observation of LV-related diseases was likewise made in bank voles.

Starting in 1989 and in the subsequent decade, young Swedish athletes, particularly orienteers, who suffered from sudden cardiac arrest and death caused by myocarditis, were the focus of much attention[184]. Since orienteering is a nature-interactive sport, a hypothesis suggesting a potential pathogen in nature, was launched. When exploring the possibility of a zoonotic pathogen, a statistical survey was conducted comparing the abundance of bank voles with the National Death Registry. The outcome showed a statistically significant correlation between the abundance of bank voles and the incidence of the diagnoses GBS and death in myocarditis (Fig. 5) and type-1 DM (Fig. 6)[28] during the time period 1971-1991.

Findings that bank voles held in captivity developed DM[185,186] prompted further studies showing that a high proportion of live-trapped bank voles *(Myodes glareolus)*, grey sided voles (*Myodes rufocanus)*, and field voles (*Microtus agrestis)* also suffer from what is appraised as DM, myocarditis and encephalitis when tested at peak densities in cyclic populations in northern Scandinavia[187]. Classic signs of DM, such as polyuria, polydipsia, glucosuria, hyperglycemia, ketosis and death were observed. Lysis of pancreatic islet β-cells and the presence of GAD-65, IA-2 and insulin autoantibodies suggested that the bank voles suffered from a type-1-like DM condition[185]. Bank voles colonized for more than a

decade were studied in more detail, and it was found that approximately 20% of these animals in captivity developed glucose intolerance, accompanied by hyperinsulinemia, increased insulin release from isolated islets, and a glucose oxidation rate consistent with type-2 DM[188]. Later on, total destruction of β-cells was also found in these colonized bank voles, similar to the pathology observed in wild-trapped voles from cyclic populations in northern Sweden. These animals thus seem to go through an initial type-2 DM-like phase with viable β-cells, evidence of insulin resistance indicated by glucose intolerance and hyperinsulinemia and later progresses to a type-1-like DM with destruction of β-cells and insulin deficiency[187,188]. Both type-1 and type-2 DM have been associated with LV infection in wild rodents, and compelling evidence suggests that infected animals develop disease when subjected to stress[187,189].

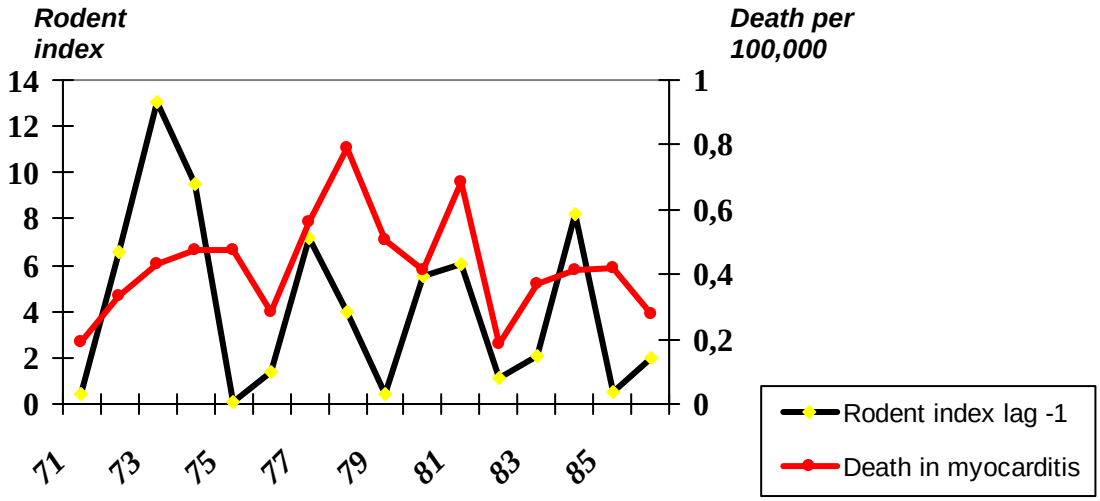

Fig. 5 *Association between death in myocarditis and vole index with a lag-1, i.e. one year delay. r=0.635 P<0.05.* [28]

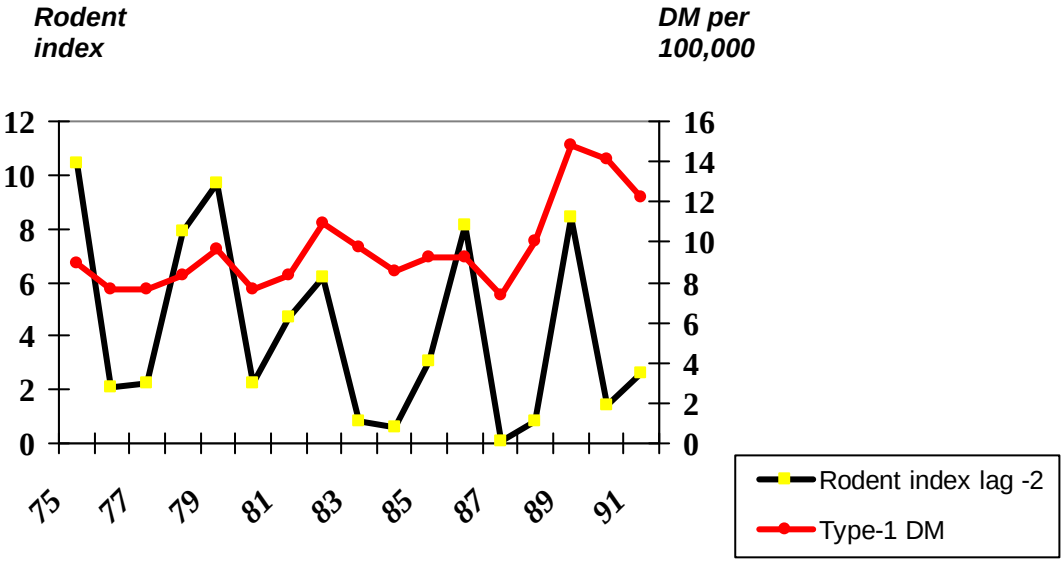

Fig. 6 *Association between incidence of type-1 DM and vole index with a lag-2, i.e. two years delay. r=0.595 P=<0.05.* [28]

Other members of the picorna virus family cause a variety of diseases in animals and humans. These diseases include the common cold, conjunctivitis, hepatitis, meningitis and paralysis. Polio virus is the

best known member of this family. Parecho virus commonly causes respiratory infections, gastroenteritis and central nervous system (CNS) symptoms in humans[190]. Cardio virus causes myocarditis in a wide range of animal species. In addition, it has been established that Cardio virus may cause diabetes and multiple sclerosis (MS) in laboratory mice[191-194]. Pigs infected with Cardio virus, however, suffer from pregnancy disorders, *i.e.* abortions and sudden piglet death[195].

Diagnosis of Ljungan virus infection

The diagnosis of viral infection in general is often done by virus isolation, detection of viral protein or viral genome, or by measuring the presence of specific antibodies. If the virus is present in sufficient concentrations, its viral proteins can be detected by methods such as ELISA. Serology is often used to detect the immune response to a viral infection. Presence of specific IgM, or a significant titer rise of specific IgG, indicate ongoing or recent infection. Progress in molecular virology has enabled the possibility of using real-time RT-PCR for the detection of viral RNA or DNA in minute amounts[196-199]. Real-time RT-PCR is a powerful tool for detecting small quantities of a virus in tissues or body fluids, such as serum or urine, by detecting specific regions of the viral genome with subsequent amplification. The same technique also opens up for the possibility of further elucidating the characteristics of the infectious agent by sequence analysis of its genome.

Immunohistochemistry (IHC) can show the presence and localization of viral antigens that may be detected by conventional light microscopy. Tissue sections labeled with antibodies that specifically react with viral-specific structures, mostly proteins, through antigen-antibody interactions are visualized with different techniques such as fluorescent dye, radioactive elements, or enzymes such as peroxidase or alkaline phosphatase conjugated to the antibodies[200,201]. IHC can thus visualize viral antigen in specific cells and in various tissues and organs, making it possible to topographically locate the virus. However, it is less useful for quantitative analyses[201].

LV is difficult to diagnose because of its inherent features. Virus isolation is as arduous a procedure in tissue cultures as in animals. Successful virus isolation has been achieved by intracerebral inoculation into one day old suckling mice, as well as in several types of tissue cultures, such as Vero cells, GMK and A-549 [27,202]. The success rate, however, is low. Virus isolation is also possible using tissue culture, but the cells must be passed for 30-50 days before any viral antigen or discrete cytopathogenic effect (CPE) can be detected[27]

2. Aims of the investigation

- To develop and evaluate an animal model for stillbirth, neonatal death and diabetes using Ljungan virus.
- To use the animal model to determine factors of importance for the development of stillbirth, neonatal death and diabetes, such as time of infection, stress and other environmental influences.
- To investigate the association between human disease during pregnancy and the abundance of the Ljungan virus reservoir using epidemiological tools.
- To explore the possible role of Ljungan virus in human and rodent fetal pathology using recently developed diagnostic tools.

3. Materials and methods

3:1 Epidemiological studies

The National Board of Health and Welfare and registers

Since 1947, all Swedish residents have unique, 10-digit national registration number, personal identity numbers (PINs), which contain the date of birth and additional four digits. The PIN is assigned to the individual immediately after birth, or after immigration. The PINs are used extensively, both by official authorities and by health care, banks and businesses. Sweden is divided into 21 counties, the administrations of which bear responsibility for the maintenance of continuously updated, computerized registers, which in turn are compiled in central registers of the total population. In these registers, the PINs are the unique identifiers. The registers receive immediate notifications upon *e.g.* birth, death, marriage, and relocation. Hence, the population registers are virtually 100 % complete and entirely up-to-date.

The National Patient Register. In 1964-1965, the National Board of Health and Welfare began collecting data on individual hospital discharges in the patient register. At discharge from hospitals, a specific form is completed for each patient, without exception. These forms are computerized locally, and the data are first stored in administrative registers kept at the hospitals and the county administrations until delivery once a year to the National Board of Health and Welfare. Each record represents one in-hospital episode. In addition to the PIN and some administrative information, including admission and discharge dates, hospital and department codes and Social Insurance Agency code, it contains medical data up to 10 surgical codes (coded according to the Swedish Classification of Operations and Major Procedures), anesthesiological procedures, and 1-8 discharge diagnoses, coded according to the 7th revision of the ICD-7 through 1968, according to the 8th revision until 1986, the 9th revision until 1996, and the 10th revision thereafter. The number of hospitals delivering data to the register has increased steadily: the register covered 60 % of the Swedish population in 1969, 75 % in 1978, 85 % by the end of 1983, and 100 % from 1987 and thereafter. Each year, the patient register includes approximately 1.7 million instances of hospital care.

The reasons for errors in the patient register are transfer errors in less than 1%, coding errors (correct diagnosis in the case record, but incorrect code given) in 6% and incorrect diagnoses in 8-11 %[203].

The Medical Birth Registry (MBR) has received standardized information on all hospital births since 1973 concerning maternal demographic data, the maternal reproductive and medical history, complications and treatments provided during pregnancy, delivery, and the neonatal period and neonatal medical conditions. Antenatal, obstetric, and neonatal data are recorded on standardized records starting with the first antenatal visit and collected until the mother and child are discharged from hospital after delivery. This Registry includes more than 99 % of all births in Sweden.

Three times in the past, the validity of the information contained in the MBR has been evaluated (1976, 1988 and 2001). Information has been analyzed on various topics for the period 1973-1998 to assess accuracy, errors and shortcomings. This evaluation started in 1999 and is not completed; still, some variables of the annual volumes can be analyzed.

Inadequacies in the registry can occur in many ways. Basic data may be insufficient, diagnoses that should have been made have not been imposed, diagnoses or other important information were not entered in the records and therefore have not been included in the registry. Details can be found in the medical journals that are not included in the information that is kept in the record. The form can raise

errors; boxes are copied into the database to go to registration. If the boxes are not correctly filled in, the information will not be saved.
Some records will not be submitted to the data. Approximately 98 % of the data on all children in Statistiska Centralbyrån, Statistic Sweden (SCB)'s birth register is in the MBR. Besides, there are a small number of cases found in the MBR but in the incorrect or incomplete identity. One reason for this may be the use of electronic medical journals. Another reason the diagnoses of the child are missing may be that the child has been transferred to neonatal care unit[51].
For information, please see: The National Board of Health and Welfare. (http://www.sos.se/epc)
The Causes of Death Register is kept by SCB and is updated with about the same delay as the Cancer Register, *viz*. 2 years. Besides date of death, the Death Register also holds information on underlying and contributory causes of death based on the ICD codes. For every deceased person a death certificate must be issued before burial is permitted. The physicians or surgeons in charge of the patient fill in the death certificates during the last hospitalization, or, for those few who die outside hospitals, by a family physician or a specialist in forensic medicine. Sweden carefully checks the national registration numbers before the data is entered into the register. The Death Register provides expected numbers of death of specific causes by calendar year, gender, and 5-year age group.

Disease incidence in humans and rodent population density

In Paper III, files from the National Environmental Monitoring Program and The Swedish National Board of Health and Welfare have been linked and computer-matched. Any temporal association between the population density of wild voles and the incidence of IUFD and GDM was investigated[18,19].

Rodent index from the Vindeln area and Västerbotten County in the north of Sweden, where rodent population densities fluctuate over the years, is considered representative for the population density of wild rodents in the cyclic regions. In southernmost Sweden (Skåne County), small rodents are present but populations are non-cyclic, whereas rodent population abundance in the north fluctuates in three to four year cycles. The analyses were performed using the fall data (end of September) and, as an index of small rodent abundance, the number of animals trapped per 100 trap nights (hereafter termed "index") was calculated.

To investigate the rodent index-disease association, a comparison was made using rodent index *vs.* the data in the national patient registry of the Swedish National Board of Health and Welfare: (http://www.socialstyrelsen.se). In Paper III, data from all hospitalization records between 1987 and 1996 were extracted. Two counties, Gävleborg and Västernorrland, with complete reporting records in the northern part of Sweden (cyclic rodents) were selected, as well as one control county in the south, Skåne (non-cyclic rodents). The international classification of diseases, 9th revision, U.S. Dept. Health and Human Services, PHS, Washington, D.C., 1988, number 9 (ICD-9), effective from 1987 to 1996, was used.

Two conditions, IUFD (diagnosis code 656E) and GDM (code 648A and/or 648W), were chosen based on observations of the effect of LV infection in animals. The incidence of IUFD and GDM was calculated as number of cases per 1,000 pregnancies (IUFD) and full term pregnancies (GDM) in that county. To increase the chances of detecting a temporal association with the rodent density in the fall, we used hospitalization admissions during the second half of each year (July-December), since the rodent densities were collected each year at the end of September. The incidence of IUFD and GDM in patients of the cyclic (northern) rodent region was subsequently compared to that of patients in the non-cyclic (southern) region. The variation between different regions concerning the two diagnoses was calculated and the number of patients per 1,000 term pregnancies was estimated for the county of Gotland (an island free of voles) and for the study region in the north (Gävleborg and Västernorrland counties), plus the entire mainland, *i.e.* Sweden except Gotland (Table 2).

3:2 Animal models

The animal models used (Paper I, II, IV and V) employed an outbred stock of CD-1 mice [ICR] BR, Charles River Laboratories, Sulzfeld, Germany. The mice had free access to water and standard laboratory chow (LABFOR R3, Lactamin, Kimstad, Sweden) with an energy content of 3.01 kcal/g. The animals were kept in individual ventilated cages (Techniplast GmbH, Germany) and maintained at a 12:12 hour dark: light cycle. The experimental procedures were approved by the Animal Ethics Committee in Stockholm and were performed in accordance with international guidelines (NIH publications no 85-23, revised 1985).

Mice were infected with LV strain *145SL*. The brains from suckling mice infected intracerebrally (i.c.) at day one with LV were used for immunization and infection. The virus stock had been passed six times in suckling mouse brain[27]. Approximately 1,000 ID_{50} were given intraperitoneally (i.p.), while control mice received an i.p. injection with the same volume of normal saline at the same time.

Intraperitoneal glucose tolerance tests (GTT) were performed by measuring each subject's body weight (BW) and administer an i.p. injection of 2g glucose/kg (as a 100 mg/ml solution). The tail of the mouse was punctured and gently squeezed to obtain one drop of blood. The drop was applied directly onto an automated glucose meter (Precision PCX; Abbott, Stockholm, Sweden [Paper I and II], (Accu Check® blood glucose meter, Roche [Paper V]). The blood glucose determinations were performed immediately prior to injection (0 min), at 60 and 120 minute post i.p. glucose injection. Normal blood glucose level of a mouse is 5-10 mmol/l. Standard Protocol/Proceedings from The Jackson Laboratory were applied.

To impose a stressful environment to the animals, the females were kept as singles and the males in shared cages (five animals per cage). Pregnant females had their BW measured three times a week and, besides the i.p. injection on GD two (virus/normal saline), they were subjected to the GTT at GD 17. An additional metabolic stress was imposed as glucose (Dextropur®) was added to the drinking water of both females and males, yielding a final glucose concentration of 100 mg/ml (10 %). Control mice were treated the same way except for an injection of normal saline. In Paper I, the impact of the stress regimen was evaluated. For monitoring adult male mice according to the stress regimen, they were kept as either singles (the non-stress regimen) or five males in the same cage (the stress regimen).

Unless otherwise mentioned, animals gave birth naturally. The delivery was constantly supervised for detection of sick or dead offspring. Dams immediately eat their sick, dying or dead pups, which makes the harvest of specimens impossible. The number of females suffering from neonatal loss, malformed progeny as well as the total number of pups borne dead or alive was recorded. The litters were kept under surveillance during the first 24 hours. The number of females suffering perinatal loss and the total number of pups borne, dead or alive were recorded. Perinatal death was defined as stillbirth or death during the first 24 hours. Fetal resorptions were defined as a positive vaginal plug, BW increase followed by a decrease of five grams or more during the last trimester and no live offspring, indicating dead embryos or that pups had been eaten by their mother. The BW increase in pregnant females normally exceeds five grams a week.

In Paper I, the effect of timing for LV exposure was studied and these groups of pregnant CD-1 mice were infected i.p. on GD(s) 4, 8, 12 or 17[122]. Additionally, in Paper I, we subjected intrauterine LV-exposed male offspring to stress. At the age of three weeks, they were put together five and five in cages according to the stress regimen. When reaching eleven weeks of age, measurements of GTT, BW, serum insulin levels, GAD-65, IA-2, insulin auto antibodies, epididymal fat and BW were performed.

In Paper II, we addressed reproductive outcome subsequent to LV exposure and its influence in combination with stress. Additionally, this Paper pays attention to mating success among adult offspring of the dams exposed to LV during pregnancy. Female mice were infected two days after conception (as described previously) and subjected to varying regimens of stress. Paper II also describes an

experiment focused on embryo resorptions in which pregnancy was terminated at GD 18. This Paper also includes a study of offspring infected *in utero*, divided into infected and non-infected groups to study reproduction success (number of offspring) and the time that elapsed between forming pairs and first litter. The period between the first and second litter was monitored. Pairs were formed with non-infected males and females, pairs with non-infected males and infected females, pairs with infected males and non-infected females and pairs with both dam and sire infected.
In Paper IV, infected male mice were used. They were injected i.p. with LV at the age of one day (as described above). When weaned at the age of 25 days, they were then put in cages with four to five males together (stress regimen). The mice were sacrificed on day 13, 17, 27, 56, 98, 130 and 174, respectively, and analyzed with LV-specific real-time RT-PCR for presence of LV RNA in various organs (brain, heart, lung, thymus, pancreas, spleen, kidney, liver and the large intestine). In this study wild bank voles, trapped in northern Sweden, were also examined for the same reason.

In Paper V, females were followed through consecutive pregnancies. Dams were subjected to three mating attempts. Non-infected male CD-1 mice were used for mating. At mating, two females shared one randomly selected male in a cage for a maximum of four days or until a positive sperm plug was registered. Males used for mating with non-infected control females were kept separate from other males.

Three groups were studied in parallel: One group (*group 1*) infected with LV i.p. in their first pregnancy on GD two, one group of offspring from the i.p. infected females (*group 2*), and the last group consisted of non-infected control females (*group 3*) followed for consecutive pregnancies. This resulted in either two or three pregnancies and the outcome of the last delivery (third mating attempt) was recorded.

Offspring of the first and second mating were removed from the dam 10 days after birth and a new mating initiated three weeks later. All females were sacrificed two weeks after the last delivery. The study endpoint was composed of an autopsy where the uterus was inspected macroscopically. Those with only one pregnancy were excluded. The numbers of implantation sites and embryo/fetal resorptions were noted. The number of females suffering from perinatal loss and the total number of pups born was noted.
Tissues (brain, lung, liver and spleen) from four of the females in *group 1* (infected i.p.), and four from *group 2* (exposed *in utero*), were collected for analysis of LV RNA by real-time RT-PCR[204]. Pups born dead were also investigated. The placenta, brain, heart/lung and liver/intestine were collected from four offspring born dead from the *group 1* (i.p. infected) females, and from five offspring born dead from *group 2* (infection *in utero)* females.

3:3 Immunohistochemistry

All 15 human IUFD cases came from central Sweden (Karolinska University Hospital, Huddinge) and were collected at autopsy (Paper III and VI). Selected cases fulfilled the criteria of IUFD, *i.e.* death occurring at gestational week 28 or later. They showed minimal autolysis by macroscopically evaluation and no specific etiology had been found by routine clinical investigation. The brain, lung, spleen, liver and placenta were sampled.

Control placenta specimens (*n*=20) (Paper III) were taken from the same area, central Sweden, as the IUFD cases and from consecutive normal pregnancies and deliveries occurring during the same time. In Paper VI, the 18 controls came from induced abortion at gestational week 15-22 because of Trisomy 21. There were also three brain samples from Boston, U.S.A. (stillbirths).

Immunohistochemical studies were performed on tissues fixed in 4 % paraformaldehyde, embedded in paraffin and cut in 4 µm thick sections[205,206]. The presence of LV-specific antigen was visualized in tissues using IHC. LV capsid proteins were stained using a rabbit antiserum against recombinant LV VP1 as primary antibody[201]. As a control to the rabbit VP1 antiserum, we used serum from a rabbit

immunized using the same protocol but with the carrier GST protein only. In addition, two different LV specific mouse monoclonal antibodies (MAb) were used as primary antibody. Normal mouse serum was used as a control for the MAbs.

Briefly, the binding of mouse MAb (diluted 1:100) or rabbit Ljungan virus antiserum (diluted 1:3000) respectively to tissue was revealed by successive incubations with biotinylated anti-IgG Vectastain peroxidase streptavidin ABC reagent (Vector Laboratories) and Vector Red substrate. Finally, slides were counterstained with methyl green, dehydrated, and mounted. Tissues from LV-infected and non-infected animals were included as controls.

Positive control specimens were also generated by mixing LV-infected tissue culture cells with non-infected cells, followed by formalin fixation and paraffin embedding. The specificity of the reaction was also confirmed by blocking the signal with LV antigen in parallel with control antigen. The primary antibodies (rabbit anti-VP1 and mouse MAbs) reacted in ELISA and IFA with LV, but not with EMCV or Parecho virus 1 and 2.

3:4 Real-time RT-PCR

In Paper IV, LV-infected male laboratory mice were examined for presence of LV-specific mRNA by real-time RT-PCR over a time of 174 days post infection. Animals were sacrificed at seven different times during the 174 day period. Samples were taken from the brain, heart, pancreas, lung, kidney, liver, spleen and large intestine. Wild-trapped bank voles were also investigated (brain, heart, pancreas, lung, kidney and liver) for presence of LV mRNA. The age of these bank voles is unknown. Control mice for this study were under the same regimen with the exception of an injection of normal saline (instead of LV) and sacrificed on day 13 and 174 post injection (p.i.).

For the study of mice in consecutive pregnancies (Paper V) tissues (brain, lung, liver and spleen) were collected for analyses of LV RNA by real-time RT-PCR. Female mice from the group infected i.p. and *in utero* were investigated.

In Paper VI, the same samples were used for IHC. All cases fulfilling the criterion of death occurring at gestational week 28 or later were included in the study. Frozen tissues from the brain, lung, spleen, liver and placenta were analyzed for presence of LV RNA. All samples came from the same area, central Sweden, as previous specimens used for IHC. Specimens to be tested by real-time RT-PCR were stored at - 70°C.

A sensitive and specific real-time RT-PCR for detection of LV RNA has recently been developed [204]. This real-time RT-PCR assay can identify and quantify LV infection in various tissues sample types using primers and two different minor groove binder probes targeting the 5' untranslated region of the LV genome. The assay, evaluated using control samples derived from various virus cultures and rodent tissues, allows precise quantification of viral load over six orders of magnitude (10^1 to 10^6 viral copies per assay) for all known strains of LV. For specificity testing, viral RNA isolated from the six different LV strains *87-012, 145SL, 174F, M1146, NY64-7855, NY64-7947* served as positive controls. Negative controls included nucleic acids from the supernatants of cell cultures infected with 20 different viruses including human Parecho virus types 1 and 2, EMCV, Theiler's murine encephalomyelitis virus, Echo virus type 30, human Adeno virus types: 2, 3, 4, 5, 9 and 40, human CMV, influenza virus types: A and B, Hantaan virus, Dobrava virus, Puumala virus, Seoul virus and Parvo virus B19 [204].

While all LV RNA samples were positive and amplicons could be isolated for sequence analysis, none of the negative controls gave a signal in the real-time RT-PCR. The positive results were confirmed by sequence analysis of the PCR products, which also allowed the Swedish and American LV strains to be distinguished.

3:5 Statistical methods

In Paper I, the GTTs were compared by Student's unpaired t-test. The stress and LV exposure studies, and also the timing of LV infection, were evaluated by one-way ANOVA followed by Scheffe multiple-comparison testing when in general statistic was significant. Due to the small sample size, ANOVA results were confirmed with non-parametric Kruskal-Wallis test. Spearman rank correlation coefficient (rho) was used for correlation with the four outcome measures of diabetes.

Fisher's one-sided exact test was used in Paper II to evaluate experiments testing offspring survival at birth, resorptions, and frequency of dead progeny in females subjected to stress.

Single classification ANOVA evaluated differences in numbers of offspring per pregnancy between treatment groups and the number of days between introductions of mating pairs and birth between treatment groups. The Bonferroni adjustment for multiple comparison tests allowed the assessment of differences between groups when the ANOVA statistic was significant.

In Paper III, a time series analysis was conducted to investigate the incidence of IUFD compared to cyclic small rodent index. Simple linear regression analysis was applied with the explanatory variable being the rodent index. $P<0.05$ was deemed statistically significant.

Fisher's exact test was used to scrutinize the human samples from fetuses and placenta and also applied in Paper V, to compare dams with live offspring to those with dead offspring and perinatal losses. Paper IV, characterizes a new real-time RT-PCR method for detection of LV and required no statistical analyses. In Paper VI, Fisher's exact test was used to evaluate the human samples, IUFD cases *vs.* controls (trisomy 21).

	Paper I	Paper II	Paper III	Paper IV	Paper V	Paper VI
Comparison between two groups:						
• Fisher's one-sided and exact test	X	X	X		X	X
• Student's unpaired t-test	X					
Comparison between three or more groups:						
• ANOVA	X	X				
- Bonferroni adjustment	X	X				
- Scheffe multiple comparison test	X					
• Kruskal Wallis non-parametric test	X					
Multivariate analysis to study association between variables Time series analyses:						
• Simple linear regression analysis			X			
Correlation between variables:						
• Spearman rank statistic	X					

Table 1. *A summary of the statistical analyses used in Papers I-VI. Paper IV, Kallies et al. characterizes a new real-time RT-PCR method for detection of Ljungan virus and required no statistical analyses. For details, please see respective studies. All P-values were considered significant if below 0.05.*

4. Results

4:1 Epidemiology

Disease incidence in humans *vs.* rodent population density

In Paper III, the temporal variation of the incidence of human IUFD was strongly associated with cyclic rodent abundance in northern Sweden, showing a very high coefficient of determination of 82 % (Fig. 7). The temporal associations were direct and no time lags were seen. Also, IUFD incidences co-varied temporally (P=0.0012, r=0.87). In contrast, there was no correlation between northern rodent index and IUFD in the south regions of the country (where the rodent population is non-cyclic).

GDM occurred with a similar incidence pattern as the IUFD, following peaks in small rodent abundance without any time lag, but this association did not reach statistical significance at P<0.05 level. (P=0.110, coefficient of determination R^2=28.8%).

In order to exclude the possibility of a nationwide temporal factor directing both rodent cycles and human disease onset, the incidence of IUFD over time in the cyclic rodent region (northern Sweden) was compared to the incidence in south of Sweden (Skåne county), where the rodent population is non-cyclic. Associations were absent between incident cases in the north *vs*. cases in the south, and were also absent between rodent index in the north and incident cases of IUFD in the south.

For the vole-free island of Gotland, the incidence of IUFD was 1.59/1,000 term pregnancies and for the entire country of Sweden 2.68/1,000 term pregnancies when Gotland was excluded. In the study region of the north, the incidence reached 3.02/1,000 term pregnancies (Table 2).

	IUFD index	Chi square one sided
Gotland (devoid of voles)	1.59	
Study area in north (cyclic voles)	3.02	P = 0.01
Entire Sweden except Gotland	2.68	P = 0.03

Table 2. *Variations between different regions in IUFD. The number of IUFD cases (ICD9 656E) over the time period 1987-1996 per 1,000 deliveries was estimated for the county of Gotland and for the study region in the north (Gävleborg and Västernorrland counties) as well as the entire mainland (all of Sweden except Gotland). Gotland is an island free of voles.*

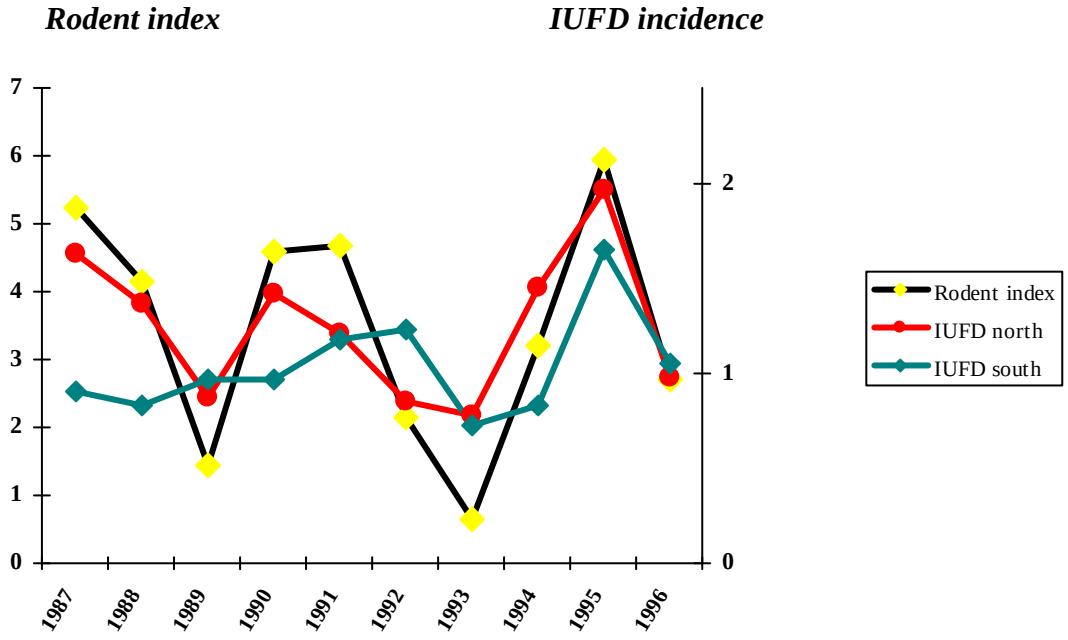

Fig. 7 *IUFD north/south vs. rodent index*
The incidence of intrauterine fetal death (number of IUFDs per 1,000 pregnancies) in two counties in northern Sweden during the autumn (July–December) of 1987–1996 compared to cyclic small rodent autumn trapping index (animals per 100 trap nights) in the same region. Simple linear regression analysis, with the explanatory variable being the rodent index, proved significant for IUFD ($P<0.001$, $R^2=0.82$).The number of IUFD per 1,000 pregnancies in the control area in the south (Skåne) is also plotted, (Paper III).

4:2 Animal models

We found that mice infected with LV during the first weeks of life developed encephalitis, myocarditis and pancreatitis as shown by histopathology. It was followed by diabetes, as measured by abnormal glucose tolerance tests 10-15 weeks after infection[187]. In Paper I, we showed that the frequency of DM varied from approximately 80 % of the male mice infected during the first three days of intrauterine life to ~ 30 % of the males infected during the second gestational week. None of the female mice acquired type-1 or type-2-like DM.

Moreover, in Paper I, we showed that viral exposure in combination with various forms of stress leads to type-2-like DM in this mouse model. The timing of the infection also mattered: The earlier in gestation the exposure, the more severe the symptoms. Furthermore, it was shown that wild bank voles caught in Sweden and their offspring, when held in captivity, developed diabetes in more than one-third of the cases. They developed type-1-like DM with destruction of β-cells as earlier described on page 10. A Danish study has previously shown that wild bank voles from Denmark developed polydipsia and stereotype behavior when kept in laboratory captivity[186].

Glucose tolerance in adult male mice was impeded by LV exposure during gestation, but only in the presence of the additional factor of stress during adulthood. The outcome of different stress-regimen groups revealed that LV only, stress only, and control treatments did not differ from each other in terms of glucose tolerance as evaluated by GTT and correlated with pancreatic morphology. The blinded morphological examination of the pancreatic glands, however, showed neither any gross structural changes of the pancreatic islets, nor any inflammatory lesions. However, in the non-infected controls the relative islet area was smaller than those observed in LV-infected mice. The islet area in relation to

pancreatic exocrine tissue, as measured with morphometry, revealed that the LV-infected mice had an expansion of islet mass compared to the non-infected control group.

In addition, BW and epididymal fat weight were examined as indicators of obesity that in turn correlates well to insulin resistance and type-2 DM. Both parameters followed the same pattern as the GTT and were significantly greater in the early LV exposure and stress group compared to the untreated control group. GTT is shown in Fig. 8.

The presence in serum of GAD-65 autoantibodies was monitored as a measure of autoimmune activity. Sera were examined from all groups included in this study and were all found to be negative. As previously described, the presence of GAD-65, IA-2 and insulin autoantibodies were detected in sera from wild-trapped bank voles. The timing of LV infection during pregnancy revealed that early gestational exposure produced an early disease onset.

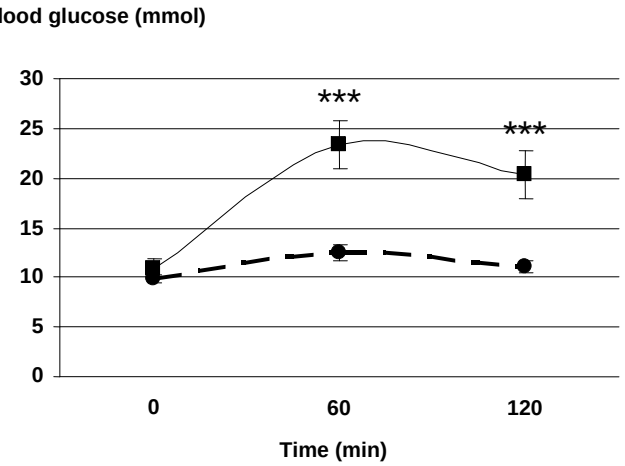

Fig. 8 *GTT comparing LV infection followed by adult stress on day four (solid line) to controls (dotted line) in CD-1 male mice at the age of 11 weeks (Paper I). Note: *** denotes P<0.001 for a chance difference vs. controls using Student's unpaired t-test.*

In Paper II we followed the reproductive outcome of LV exposure, the combination with stress and LV, as well as mating success among the adult offspring (both dams and sires) of the dams exposed to LV during pregnancy. This resulted in six out of seven infected females giving birth to a total of nine stillborn pups and fetal remains, while one dead pup was present in the non-infected and stressed group. There was no difference in the number of pups per litter or in BW between the group of infected animals and controls. The proportion of pregnancies with dead pups of infected dams was significantly greater, 86 % (6 of 7), than that of the non-infected controls, 14 % (1 of 7), (*P*= 0.029, analyzed with Fisher's one-sided exact test). Both LV-infected animals and controls were subjected to GTT at GD 17 and showed no signs of GDM in any group.

Paper II, investigated the presence of embryo resorptions/implantation sites. A total of six fetal resorptions/implantation sites were noted in four infected females, while no fetal resorptions/implantation sites were noted in the four non-infected dams. Regarding malformations, we found that one pup in the infected group had a malformation of the head, exencephalia, and displayed eight pairs of ribs. In addition, one infected and two non-infected pups were randomly selected for histopathological examination. The randomly selected infected pup showed no visible gross malformations. However, histopathology revealed that this fetus displayed six pairs of ribs and that the dorsal fat depots were markedly reduced in size. One adrenal gland, one kidney, the cerebellum, spinal cord and one eye were missing in this animal. No lesions were found in the two randomly selected non-infected pups.

In addition, *in utero* LV exposure in combination with stress resulted in significant perinatal death, as five out of eight females delivered dead offspring and a total of 13 stillborn pups. Only one dead offspring was recorded in the non-infected and stressed control group.

Histopathological examination of eight offspring born dead from three different infected and stressed dams revealed malformations of the head and brain and one pup had an external hydrocephalus.

Histopathological examination was also performed on six surviving suckling mice sacrificed one week after birth with clinical signs of encephalitis, *i.e*. involuntary movements. The encephalitis was associated with intense basophile and hypercellularity in the vascular endothelial lining of the brain blood vessels.
Myocarditis and pancreatitis were also found in some individuals.
The continuing perinatal problems (Paper V) of LV-exposed pregnancies imply that complications persist. There were no differences between the i.p.-infected group and the vertically exposed females when looked upon during consecutive pregnancies. Of the i.p. infected dams, 11 out of 13, suffered neonatal deaths, whereas the congenitally infected females had dead offspring in 4 out of 20 cases. Frequencies of resorptions were one in the i.p. group and four in the vertically infected group. None of the control animals experienced any problems of this kind (Table 3).

	Total no. of females	Pregnant	Pregnant delivering dead pups	Pregnant Loss of entire litter	Total no. of dead pups	Pregnant with fetal resorption
Infected i.p.	15	13	10	1	>21	1
In utero exposed	20	20	4	1	>15	4
Controls	15	9	0	0	0	0

Table 3. *Females infected i.p, exposed to Ljungan virus in utero and controls followed during pregnancy, (Paper V).*

4:3 Immunohistochemistry

In Paper III, ten, and in Paper VI, five cases with the diagnosis of IUFD were evaluated by routine histopathology and by LV-specific MAb analyses. Data on gestational week of stillbirth, observed maternal disease and pregnancy complications, autopsy findings as well as information on placenta pathology are given in Tables 4 and 5. Histological interpretation was difficult as tissue degradation of various degrees was observed in all organs. However, the brain showed a clear and distinct IHC pattern for LV in IUFD patients. In Paper III, four out of ten (Table 4), and in Paper VI, all five samples in at least one organ (Table 5) turned out positive for LV when analyzed blindly by two different examiners.

In addition, tissue from the liver, heart, pancreas, placenta and thymus was also investigated for the presence of LV antigen. Positive IHC staining was also seen in other organs. However, the pattern herein was less distinct and often located at sites of tissue degradation/autolysis.

The placenta showed positive staining in five of the 10 cases, including all four cases with positive IHC staining in the brain tissue. None of the 20 control placentas were positive in this blinded analysis (Paper III). In Paper VI, the placenta was positive for LV in three of the five specimens available while only one of the 18 control brain specimens scored positive, (P=0.01, Fischer's exact test). In addition, in Paper VI, the three samples from Boston were analyzed by IHC, using the same technique, revealing that one of the three brain tissues was positive for LV. *Nota bene*, this case describes a stillbirth in gestational week 15.

Case nr	Sex	Gestational week at stillbirth	Maternal disease	Pregnancy complication	Autopsy findings	Placental pathology	Etiology of stillbirth	IHC brain	IHC placenta
1	F	40	not stated	twin	signs of asphyxia	small placenta, diamnio-dichorio, acute chorionitis, hypocoiling	unknown	Negative	Positive
2	M	38	no	no	suspicion of asymmetric growth restriction, hypolobation of the lung, signs of asphyxia	small placenta, accessory lobe, long cord, arteriopathy in decidua	placenta insufficiency (probable)	Positive	Positive
3	M	37	not stated	twin, growth restriction 27%	cerebral infarction, intraventricular hemorrhage	small placenta, diamnio-dichorio, abruption, chronic villitis 30%, single umbilical artery, hypocoiling	abruption (probable)	Positive	Positive
4	M	37	no	no	signs of asphyxia	small placenta, chorangiosis, thromboses in fetal vessels in chorionic plate	placenta insufficiency (possible)	Negative	Negative
5	F	term	not stated	no	signs of asphyxia	large placenta, villus edema, minor infarction, signs of hypoxia	unknown	Negative	Negative
6	M	42	not stated	no	slight facial dysmorphism, signs of asphyxia	small intervillous thromboses, chronic villitis 2%, chorangiosis	unknown	Positive	Positive
7	M	31	no	minor bleeding before stillbirth time	suspicion of symmetric growth restriction, signs of asphyxia	small placenta, widespread chorangiosis	placenta insufficiency (possible)	Negative	Negative
8	M	term	over weight, borderline hypertension	gestational diabetes (insulin treated)	signs of asphyxia	small placenta, old abruption with secondary infarction, signs of hypoxia	abruption (probable)	Negative	Negative
9	F	41	no	no	signs of asphyxia	small placenta, infarction 2%, widespread signs of hypoxia	placenta insufficiency (probable)	Positive	Positive
10		33	no	no	signs of growth restriction, signs of asphyxia	abruption 35%, infarction 50%, chronic villitis 20%, hypocoiling	abruption (probable), placenta insufficiency (certain)	Negative	Negative

Table 4. *Ten cases of IUFD investigated for LV presence by IHC, (Paper III).*

Case nr	Gestational week	Maternal disease	Pregnancy complication	Autopsy findings	Placental pathology	IHC	Real time RT-PCR
1	41	No	No	secondary signs of asphyxia, no other abnormalities	signs of infection, solitary infarction and intervillous thrombosis, velamentous cord	Pos placenta	Pos (spleen)
2	28	not stated	not stated	no abnormalities	marginal cord, otherwise no abnormalities	Pos placenta	Neg
3	39	psychiatric illness	no	ectopic thymus, calcification in CNS, otherwise no abnormalities	large placenta, maturation defect in villi	Pos CNS	Neg
4	39	No	suspicion of infection	dysmorphic facies, simian crease, hyperlobated lung, supernumerary coronary artery, hypoplastic uterus, hydroureter	small placenta, maturation defect in villi, signs of hypoxia	Pos CNS and placenta	Pos (CNS)
5	41	No	no	dysmorphic facies, hyperlobated lung, ventricular septum defect	small placenta, maturation defect in villi, chorangiosis, signs of infection, fetal thromboses, hypocoiled cord	Pos CNS	Pos (lung)

Table 5. *Five cases of IUFD investigated for LV presence by IHC and PCR, (Paper VI).*

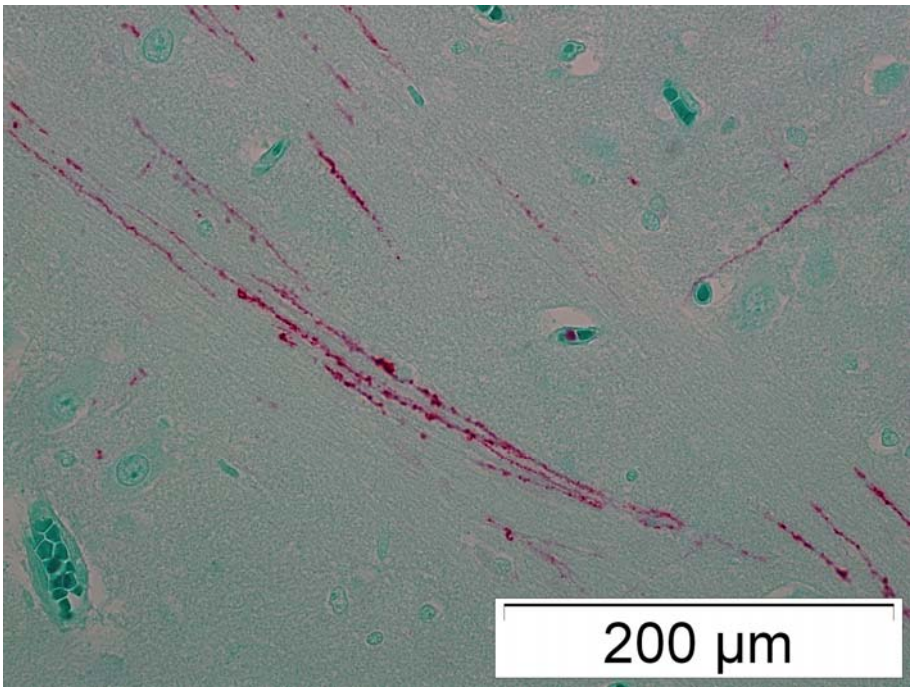

Fig. 9 *Brain tissue from a case of IUFD stained with LV specific monoclonal antibody (red staining). The picture shows staining of neurons in the parenchyma. Routine pathology found no sign of inflammation in this tissue.*

4:4 Real-time RT-PCR

In Paper IV, all the organs taken from non-infected control mice tested negative for LV-specific RNA, as evaluated by real-time RT-PCR. All samples were additionally tested for murine hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA that served as a housekeeping gene to examine whether the RNA extraction was successful. All samples included in the study were positive for murine HPRT mRNA, thus validating the RNA extraction process. All organs from infected animals were found positive for LV by real-time RT-PCR. In the acute infection, the highest levels of virus genome equivalents were found in the brain followed by pancreas and heart. In all organs, levels of LV genome equivalents decreased and reached a minimum at day 56 p.i. By day 56 p.i., LV RNA levels began to increase in all organs and remained at a constant level from day 98 p.i. until day 174 p.i. when the experiment was terminated. In this study, wild bank voles (*n*=13) were also tested for presence of LV RNA. A total of nine animals were found positive for LV by real-time RT-PCR in at least two organs, while one was positive in all examined organs. The highest mean LV genome copy number was found in the brain.

In Paper V, the real-time RT-PCR test was positive for LV in one or more organs, in three out of four cases from the *group 1* dams (infected i.p.), while one out of four females exposed to LV *in utero* was real-time RT-PCR positive (Table 6 A). Real-time RT-PCR testing on the dead offspring revealed that in those from the *group 1* dams (i.p.), three out of four were found positive for LV and in the *group 2,* pups (*in utero* exposure), three out of five were real-time RT-PCR-positive in one or more organs (Table 6 B). None of the infected dams were positive for LV in the brain samples (Table 6 A). In contrast the offspring was found LV-positive by real-time RT-PCR in the brain samples (Table 6 B).

The analyses of LV RNA in human tissues in Paper VI, revealed that three of the five IUFD patients were positive in at least one organ (Table 7). One case (*no* 4), was positive for LV with both real-time RT-PCR and IHC in brain tissue (Table 7).

Females from *Gr. 1* and *Gr. 2*	Brain	Lung	Liver	Spleen
Nr. 1 Infected i.p.	neg.	pos.	neg.	pos.
Nr. 2 Infected i.p.	neg.	neg.	neg.	neg.
Nr. 3 Infected i.p.	neg.	neg.	pos.	pos.
Nr. 4 Infected i.p.	neg.	pos.	pos.	pos.
Nr. 5 Exposed i.u.	neg.	neg.	neg.	pos.
Nr. 6 Exposed i.u	neg.	neg.	neg.	neg.
Nr. 7 Exposed i.u	neg.	neg.	neg.	neg.
Nr. 8 Exposed i.u	neg.	neg.	neg.	neg.

Table 6. A *Real-time RT- PCR for LV, from females, Group 1(i.p. infected) and Group 2 (in utero exposed), [Paper V]*.

Pups from females, *Gr. 1* and *Gr. 2*	Brain	Lung/heart	Liver/intestine	Placenta
Nr. 1 Infected i.p.	pos.	n.d.	n.d.	n.d.
Nr. 2 Infected i.p.	pos.	n.d.	pos.	pos.
Nr. 3 Infected i.p.	pos.	neg.	pos.	n.d.
Nr. 4 Infected i.p.	neg.	neg.	neg.	n.d.
Nr. 5 Exposed i.u	neg.	n.d.	neg.	n.d.
Nr. 6 Exposed i.u	pos.	n.d.	pos.	pos.
Nr. 7 Exposed i.u	pos.	n.d.	n.d.	n.d.
Nr. 8 Exposed i.u	pos.	neg.	neg.	neg.
Nr. 9 Exposed i.u	neg.	neg.	neg.	n.d.

Table 6. B *Real-time RT-PCR for LV, offspring from females, Group 1(i.p. infected) and Group 2 (in utero exposed), [not done, n.d], (Paper V).*

	Tissue	IHC pos	IHC neg	PCR pos	PCR neg	Correspondence
Case 1	Brain					
	Spleen		X	X		
	Lung					
	Placenta	X			X	
Case 2	Brain					
	Spleen					
	Lung					
	Placenta	X			X	
Case 3	Brain	X			X	
	Spleen					
	Lung					
	Placenta					
Case 4	Brain	X		X		X
	Spleen					
	Lung					
	Placenta	X			X	
Case 5	Brain	X			X	
	Spleen					
	Lung		X	X		
	Placenta					

Table 7. *LV presence in five IUFD cases analyzed by real-time RT-PCR and IHC, (Paper VI).*

5. Conclusions

- We successfully developed a mouse model for studying stillbirth, neonatal death, diabetes and viral persistence *in vivo* using Ljungan virus.
- It was found that a type-2 diabetes-like disease can be induced by intrauterine Ljungan virus infection in combination with postnatal stress in this mouse model. Moreover, Ljungan virus infection in combination with stress resulted in a high frequency (>50 %) of IUFD, malformation and neonatal death.
- We found a significant association between the abundance of the natural Ljungan virus reservoir (rodent index) and IUFD in the northern part of Sweden, but not in the south.
- We detected Ljungan virus with immunohistochemistry in brain tissue and placenta of some human IUFD cases, whereas none of the placentas from normal pregnancies and one of the Trisomy cases showed positive immunohistochemical staining for Ljungan virus. We succeeded in the development of a real-time RT-PCR method for detecting presence of Ljungan virus genome in human fetuses as well as in animals.

6. Discussion

This thesis examines the role of LV as a possible pathogen, both in human fetuses and newborn infants as well as in animals, with regard to perinatal mortality and diabetes. We propose the possibility of a rodent-borne zoonosis, supported by the findings of LV being strongly associated with reproductive disorders and type-1 DM in epidemiological investigations. The fact that diseases has been found in wildlife, in conjunction with the possibility to transmit LV in laboratory animals, causing similar diseases thus fulfilling Koch's postulates under controlled conditions is estimated as one component of the evidence. This is demonstrated in the time-series analysis, where a correlation between the incidence of IUFD and rodent population densities in the north of Sweden is seen (Paper III). Furthermore, the fact that LV can be propagated in laboratory animals, causing similar disorders as in humans, adds further credence to our proposal, as well as the presence of LV RNA and antigen in human tissue samples from deceased fetuses.

Many viruses, bacteria and/or protozoa have been associated with stillbirth but few have shown to be etiologically causative in developed countries[68,76]. The findings presented in this thesis challenge this view, and we propose that LV may be etiologically related to some cases of human IUFD. This bears striking resemblance to clinical suspicions that obstetric disorders like preterm birth, IUGR and pre-eclampsia could be caused by infections[118]. The epidemiological correlations reported here, the supportive information from the native mammalian fauna and the experiments with laboratory mice, along with the detection of LV by IHC and real-time RT-PCR in pathological human tissues, in their entirety suggest a specific infectious etiology involved in various disease outcomes of pregnancy. Diagnostic tools for LV detection with high sensitivity and specificity for application to clinical samples are constantly under development.

6:1 Epidemiology

We hypothesized that the abundance of voles and other small rodents harboring LV might be reflected in the incidence of human morbidity and mortality. The temporal variation of the incidences of diseases suggests a relationship with rodent abundance in northern Sweden. The epidemiological studies supply statistical support for co-variation with rodent density and show high coefficients of correlation. Our findings provide epidemiological links between two important diseases of pregnancy, IUFD and GDM. GDM is quite prevalent, its incidence exceeding 5%, and stillbirth incidence is 0.5-2% in most developed countries. Stillbirth remains one of the most devastating failures of pregnancy.

Fig. 10 *Bank vole with a two week old offspring.* *Foto: B. Niklasson*

The time series analyses presented provide strong correlative support for an association between IUFD incidence and rodent population densities in northern Sweden. This emphasizes the possibility of a rodent-borne zoonosis. The intriguing possibility that LV might be an underlying agent responsible for these conditions of human reproduction, both of unknown cause, deserves careful consideration. We put forward that a single agent might be responsible for a substantial proportion of cases of IUFD, and that this agent may be the LV. The findings that disease found in wildlife can be transmitted in laboratory animals is suggested to be evidence of LV being involved in diabetes and reproductive loss (Papers I, II). In this thesis, the association between GDM and rodent abundance, while suggestive, did not attain statistical significance at 95 % confidence interval. This might be due to the shorter duration of GDM records, and thus lower statistical power or a factual lack of correlation.

We do not believe that the epidemiological studies reported here include any systematic errors falsely creating the rodent density-disease incidence association. Conversely, we do believe that our study, using information from a national database, might include non-systematic errors. Incorrect ICD coding of clinical conditions is one such potential error. In a study performed by the National Board of Health and Welfare, the overall proportion of incorrect or missing PINs was 7 % in 1977, but it dropped to less than 2 % in 1983. The proportion with invalid diagnostic codes or procedure codes (codes not listed in the code-books) increased from 0.4 % in 1964-1969 to 0.5-1.7 % during the 1970s. The reasons for errors in the inpatient register are transfer errors in less than 1%, coding errors (correct diagnosis in the case record, but incorrect code given) in 6% and incorrect diagnoses in 8-11 % [207,208].

The finding that the same fluctuation pattern of disease incidences in the north did not occur in the south is important, as this shows that the northern disease pattern linked to the vole cycles in that area does not occur nationwide. Also, the absence of significant correlations of disease incidences in southern Sweden with those in northern Sweden shows that these rodent densities are influential only in the region in which native rodent population cycling occurs, and not nationwide. This is highly suggestive of a contagious cause of these diseases.

6:2 Viruses

Reproductive loss, as well as malformations during pregnancy in mammals, has previously been associated with infectious agents, *e.g.* syphilis, toxoplasmosis, rubella, CMV, HIV, parvo-B19, entero viruses, LCMV and HSV, just to mention a few [209-211].

One example of a LV-related virus is Theiler's murine encephalomyelitis virus (TMEV), a naturally occurring murine picorna virus similar to that of polio virus[212]. It persistently infects the central nervous system of mice. TMEV belongs to the cardio virus group. The natural host is mice and in the wild the virus causes gastrointestinal infection followed by infection of the nervous system. TMEV comprises two main groups, one of which produces acute fulminant encephalomyelitis, fatal within approximately one week, the other a chronic persistent central nervous infection, along with demyelinating lesions of the spinal cord. A classic study by Theiler in 1934 showed that infected mice may not show symptoms and still shed the virus, which is easily transmitted through soiled bedding[176].

Another member of the genus cardio virus causes a clinical disease with high mortality in young piglets: EMCV causes intrauterine and postnatal deaths in pigs[195, 213-216].The observation that piglets sometimes die with no histopathological lesions resembles the hurdles of LV detection in tissue and the fact that experimentally LV-infected pregnant mice sometimes die during the perinatal period (Paper II, IV).

A third example of a murine model is LCMV, a rodent-borne zoonosis that is reminiscent of LV and is known to be an under-diagnosed fetal teratogen[24,112]. This virus causes neonatal hydrocephalus in humans. These conditions are sometimes misdiagnosed as various chromosomal, ophthalmologic and neurological syndromes. There are less than one hundred reported cases of LCMV worldwide, half of them from the U.S.A.[217,218]. LCMV is shed in urine, feces, semen, nasal secretions, saliva and milk by rodents. Human infection is usually acquired by direct contact with excrement contaminated with infectious virus or by inhaling aerosolized virus. Human-to-human infection is not documented other than in organ implants[112,218-221]. LCMV in pregnant women causes fetal hydrocephalus, micro- or macrocephaly, intracranial calcifications, chorioretinitis and non-immune hydrops[222]. Mice are known to carry silent, vertically transmitted LCMV infection[218] as we propose in Paper V is the case with LV. The finding that LV is associated with lethal CNS malformation and anencephalia, as well as hydrocephalus, in mice is also an important observation that needs further exploration (Paper II). Virus was detected in feces of male mice subjected to stress for three months, indicating the importance of environmental factors such as stress for viral activity as well as suggesting one potential mode of transmission (Paper IV).

LV has been found in a wide host range, including several species of native rodents[27,223], and LV isolates have also been found in wild rodents of North America[223-225]. The presence of LV in at least two continents in various species, and the recent suggestion that LV may be close to the root in the picorna virus family based on phylogenetic analysis, suggests that this virus may be widely distributed[223,226].

The fact that LV grows in low titers and produces a weak antibody response poses problems in terms of accurately detecting its presence. Further investigations directed towards gaining a deeper insight and understanding of the molecular mechanisms concerning the regulation and specific role of viral proteins in modulating LV-host cell interactions are clearly warranted[227,228].

A feasible theory to the hurdles encountered in the detection of LV in tissue and serum could be that, depending on whether there is a persistent infection, the virus may hide within cells and not present itself with detectable proteins, free RNA or/and serum antibodies in sufficient concentrations, which would make it very difficult to identify by the diagnostic tools currently available[229].

The fact that LV RNA was detectable in female mice six months post viral i.p. inoculation suggests a persistent infection (Paper V). Presence of viral RNA in the brain of dead offspring, supports the hypothesis that LV infection acquired *in utero,* and in combination with maternal stress, can cause perinatal death and that the replicating virus is an important component in the pathogenesis.

What can be said about the route of transmission of LV to the human host? Many picorna viruses are transmitted by the fecal-oral route. Direct contact with small rodents, or material contaminated by rodents, is one possible route of transmission. While small rodents and especially the bank vole may constitute the main source of viral replication, the wide host range of LV and the lack of a clear urban/rural gradient in disease incidence suggest that transmission to humans is likely more complex. In an attempt to shed light on this, we investigated the hospital database of Swedish Gotland, an island with several species of small rodents but no bank voles. The incidence of IUFD was found to be approximately half of that of our northern study area and of the entire mainland of Sweden (described on page 18). This suggests that voles may be an important, but not exclusive, source of LV for human infection in Sweden. Future studies of possible routes of viral transmission are clearly required.

6:3 Animal models

Regarding diabetes, there are pertinent observations from our animal data. The animal data presented suggests that LV can induce both type-1 DM and type-2 DM symptoms, depending on the species and the phase of disease within a single species. Type-2 DM, with its failing glucose regulation, high insulin output, body fat accumulation and insulin resistance, could be the first stage of the diabetes. Later on type-1 DM, with its loss of insulin production capacity and β-cell death, emerges as the end stage of a single pathogenic course. Type-1 DM could have a mean incubation time of two years, according to our previous studies (Fig. 6). The lighter and typically temporary manifestation of GDM with its abnormal glucose tolerance becoming apparent shortly after infection may be part of the same underlying pathology, perhaps precipitated by the diabetogenic stress of pregnancy[149].

The possible role of stress in disease outcome following viral exposure deserves to be taken into consideration. It has been suggested that different stress factors may influence disease outcome and that infection can be an important feature[230,231]. Our experiments in laboratory mice show that pregnant dams subjected to the combination of stress and LV exposure have the worst outcome in terms of both fetal deaths and malformations (Paper II).

In humans, a role for both psychological stress and the general physiological stress of pregnancy in diabetes induction has been proposed[145,232-234]. It is possible that that the combination of stress and viral infection may be playing a similar role in humans as that demonstrated in the rodent models currently studied. One possibility as to how the stress factor affects the pregnant woman and her unborn child has been addressed herein, highlighting the influence of intrauterine environment[235]. It may be possible

that, in combination with infection, psychological/physiological stress during pregnancy can affect the outcome of pregnancy as well as the growing child or adult[236].

Our studies also suggest that LV RNA persists in CD-1 laboratory mice. It has previously been shown that RNA viruses may persist in the central nervous system[237-239]. This seems true also for LV, as shown in male mice (Paper IV).

In their entirety, our findings demonstrate that LV causes a systemic infection that occasionally may lead to perinatal disorders or diabetes later in life when infected vertically or during the first week of life. We suggest that disease onset may be influenced by LV infection in combination with environmental factors. Such a factor seems to be the influence of stress. LV infection in early gestation required the combination of adult stress to produce the outcome of glucose intolerance, increased body fat, hyperinsulinemia, and - for bank voles - auto antibodies against islet antigens. The timing of exposure also influenced the later diabetic outcome; the earlier, the more severe the symptoms. Stress combined with LV infection was also essential for stillbirth in the animal studies. In light of these findings, it seems possible that voles at peak density are, besides being infected with LV, at a high stress level. This may collectively lead to the major casualties among the rodents when at high population numbers.

The studies in this thesis also illustrate the importance of constantly monitoring the delivery phase to gain crucial information. While malformed offspring and resorptions can be diagnosed by sacrificing the pregnant mice two to three days before expected delivery, this procedure will not necessarily identify all pups that will die perinatally. In addition, offspring born dead or dying soon after delivery are frequently eaten by the mother along with their placentas. Complete and comprehensive information can thus only be obtained by continuously observing the animals during the entire period of expected delivery and thereafter.

This study employs a mouse model for investigation of IUFD and the incidence of diabetes in offspring of infected dams (Paper I, II, V). The use of an outbred stock, CD-1, has the advantage to reflecting better than in-bred mice what may occur in the “outbred” human population, although we clearly realize the difficulties and ambiguities in extrapolating animal results to human pathology. A disadvantage may be the relatively low number of animals used. To attain statistical power, a larger sample size may be required. The number of animals used in these experiments has been minimized due to ethical and economical considerations, and we have still been successful in developing murine models that seem to be reproducible. The phenotypic and genetic variations are for obvious reasons greater in an outbred stock, and the degree of genetic variation between individual colonies could be so pronounced that it may, conceivably, be difficult to reproduce previous results from experiments on other colonies. An optimal strategy may be to use more than one inbred strain[240]. On the other hand, it is known that inbred strains of mice may differ in their susceptibility to infections and demonstrate greater variability in the type of immune response they generate upon exposure to pathogens[241]. Other potential sources of errors in animal experiments may be the influence of circadian fluctuations and the importance of the external environment for the outcome of the study[242-244]. Possible differences in food intake between individuals and whether this may have influenced the outcome are also worth mentioning.

Another source of error in animal models could be other pathogens that interact in different ways with the infected group. It is well known that mice may carry several infectious agents, *e.g.* Parvo and Rota virus, TMEV, mouse hepatitis virus (MHV) and pin worms. The mice in our experiments all came from Charles River Laboratories, Germany[241]. Although they are regularly screened for the above mentioned pathogens (and others), there is always the possibility of a contaminated delivery of mice although we have no evidence in favor of this. Our animals were kept in individually ventilated cages, which severely inhibit cage-to-cage transmission. The animal studies clearly show that control mice, kept under similar conditions as the LV-treated mice, did not suffer from perinatal complications or glucose intolerance[241,245].

We have not in the murine models of IUFD in this thesis, been successful in detecting virus presence in placenta. The inflammatory response in the placenta could be weak in response to LV as is the case with *L. monocytogenes*; still it may eventually lead to spontaneous placental death and poor pregnancy outcome[124].

6:4 Diagnostics

New pathogens require new diagnostic tools to determine their role as a potential etiologic agent. However, it should be noted that diagnostic assays for novel infectious agents go through an initial phase where large clinical studies using the assay are necessary to determine its sensitivity and specificity.

Both the IHC and the real-time RT-PCR assays for LV have been thoroughly evaluated and do not react with other picorna viruses[204]. Evaluation of the two methods in animals infected under controlled conditions shows that the methods can be used to prove the presence of LV infection. However, negative results cannot be used as definitive proof of the absence of infection, as low viral titers may escape detection. Additionally, it is possible that currently unknown different strains of LV may, or may not, be reactive in the assays presently used. The fact that LV has been detected in several locations in Europe and in the U.S.A. suggests a worldwide distribution[224]. This is supported by the findings of LV-positive IHC staining in one of the stillbirth brain sample from Boston.

The real-time RT-PCR that is now available makes it possible to detect the presence of LV RNA as indirect proof of infectious virus. The presently existing method requires tissue specimens to be stored at low temperature (–70°C). Unfortunately, a limited amount of such specimens were available for the studies in this thesis. Specimens that have the appropriate collection and storage characteristics for real-time RT-PCR testing are currently being actively sought for future studies[204].

Although the biological characteristics of pathogens will influence the possibility of their detection, this decisive factor does not necessarily reflect their importance as etiologic agents.

The IHC data presented herein is considered as one piece of evidence that LV is present in humans, and in particular that it is associated with disease. The assays used to detect the presence of LV, or LV immunity, are based on recently developed diagnostic tools. The validation of such tools often takes advantage of established standards as controls. In the case of a novel virus, this is initially very intricate and poses special problems. In spite of this, it is our belief that the human data presented – derived from a combination of assays - strongly suggest that LV may infect humans. The findings, although based on limited numbers and a somewhat scattered collection of specimens, also show a consistent link to investigated diseases, which suggests direct LV causation. Furthermore, the laboratory results are in line with expectations from the animal models, including variations in the amount of virus found over the course of a disease.

Positive IHC staining provides evidence for the presence of LV in a sample, as the specificity of the MAb test is considered specific (Niklasson, unpublished observation). As mentioned before, however, negative IHC cannot be used as evidence for the absence of virus. Factors that might contribute to such a false negative effect include low viral titers and *post mortem* autolysis, contributing to the proteolytic destruction of viral antigens. For these reasons, it appears appropriate to consider the proportion of samples positive for LV by IHC as a minimum estimate of viral prevalence.

The cases of IUFD investigated showed LV presence in the placenta and the virus was also detected in the brain of the stillborn fetuses. The fact that histopathology revealed no signs of meningitis or encephalitis (Paper III), is consistent with observations of LV-infected mice developing diabetes. These animals had impaired glucose tolerance, with virus present in the insulin-producing islets as detected by IHC, but showed no local inflammatory islet reaction[246,247].

In the study of female mice in consecutive pregnancies (Paper V), viral RNA was also detected in one of the four females tested that had been exposed to viral infection *in utero*. This finding, in conjunction with the observation of real-time RT-PCR-positive dead offspring, suggests that some of these females also suffer from a persistent LV infection. No histopathological analyses were performed on the females or their offspring. The limited number of specimens available for real-time RT-PCR analysis unfortunately precludes any conclusions neither as to the presence of virus or virus load in any particular animal or organ nor as to the outcome of the pregnancy.

Because LV requires a relatively long adaptation time in tissue culture, viral isolation is difficult. Serology is another common tool for diagnosing previous or present viral infection. However, the antibody response to LV in animals varies, but is usually weak or absent in chronically infected animals. Animal experiments also show that several weeks or months may elapse between acute infection and the onset of disease[127,246]. At the time of disease onset, the viral load measured indirectly by real-time RT-PCR may be low and often close to the detection limit of this assay[171, 248]. In contrast, infected cells may contain relatively high levels of viral protein detectable by IHC. The precise nature of the pathogenesis of target damage induced by LV infection remains to be investigated. Our preliminary data (Papers IV and V) suggests a long-lasting or chronic infection. The observations from IHC and histopathology both suggest vascular tropism[200,201]. This may be important, especially since LV seems to infect different organs, *e.g.* muscles, glands and brain.

6:5 The possible effect of Ljungan virus in fetal pathology, obstetrics and diabetes

Exact knowledge of the cause of death is essential for reducing the incidence of such cases in the populations and for correct treatment in future pregnancies. The proportion of cases in which no underlying cause is identified varies between 9 and 50 percent in the literature. A study by the Karolinska Institutet has shown that the percentage of unexplained cases may be reduced if a relevant and comprehensive investigation is carried out in all cases of intrauterine stillbirths[249]. Several studies have shown that the examination of the placenta and a fetal autopsy are investigations contributing to an explanation of these cases[250,251].

Analysis of infection is suggested as a regular procedure in a Swedish study. Based on the research conducted here, it is recommended that LV be included in the panel of infectious agents screened. Currently, besides screening for different infectious agents, analyses include chromosomal aberrations, coagulation disorders, feto-maternal transfusion, intrahepatisk cholestasis, placenta pathology, and fetal autopsy[250]. Causes and risk factors in stillbirth vary among different populations. Continuous data collection and evaluation of results in cases of stillbirth will help lead to improved understanding of outcomes. New findings on the etiology of fetal death and more advanced diagnostic procedures will lead to important improvements in this field of medicine. Since LV has been identified on different continents, studies on the intrinsic genetic variation of the virus must be pursued. In addition, the inclusion of LV in the diagnostic arsenal of tests both for IUFD and fetal malformations seems essential. It is possible that LV diseases differ depending on the geographic location and rodent abundance.

An infectious etiology has been suggested for type-1 DM. Several viral agents have been proposed, based on either observations of virus associated with disease in humans or the fact that virus can induce diabetes in laboratory animals. Rubella virus, CMV, varicella zoster and cardio virus have all been circumstantially implicated[252-254]. Most attention has been paid to Coxsackie B viruses, where the virus has been recovered from pancreas of patients with recent onset type-1-DM[255]. Coxsackie B virus can induce diabetes in some strains of laboratory mice using specific strains of virus[132,256]. Seroepidemiological studies in Finland first suggested that Entero virus infection during pregnancy could result in diabetes in the offspring[257-259]. A later study, employing large sample sizes and focusing on early onset type-1 DM, however, failed to confirm this hypothesis[258]. Epidemiological and clinical studies have not yet yielded unambiguous and conclusive information as to whether any of the viruses

mentioned above can induce diabetes in humans. We hypothesize that LV may account for a proportion of type-1-DM. We base this suggestion on an assessment of the complete range of evidence generated and summarized in this thesis. The fact that disease found in wildlife and associated with LV can be transmitted in laboratory animals, thus fulfilling Koch's postulates under controlled conditions, is one component of the evidence. Previous and current studies also suggest an epidemiological link between the suspected reservoir/vector and human disease incidence, the human diseases involved, laboratory evidence of LV from patients, and studies pointing to LV in human disease (Paper I).

LV creates a persistent non-cytopathogenic infection in a wide variety of tissue cultures and the available evidence suggests that it may cause a long lasting or chronic infection in laboratory mice developing late onset disease (Papers IV, V) [27] Based on the experiments on mice infected shortly after birth, an infectious sequence of high virus load may be followed by near disappearance of the virus despite its generating diabetes only becoming evident weeks after viral exposure. The association with stress, and the course of LV infection as shown in our studies, resembles many clinical observations on stress and diabetes in humans[230,232,234,260,261].

6:6 Further studies

The pathway leading to IUFD should be further explored and various risk factors, early signs, diagnostics, prevention and possible etiological agents identified. We suggest that infection is the lowest common denominator in the majority of unexplained cases of perinatal death, IUGR, preterm birth as well as pre-eclampsia. This is also coincident with the explanatory factor of 80 % from the epidemiological investigation. We imply that LV or some other pathogen also carried by the rodents may be of epidemiologically consequential significance.

The association of LV with neonatal deaths in animal models has been clearly demonstrated and our work also underscores the important biological consequence of stress. The interaction between viral infection, stress and the intrauterine environment as determining factors in programming for adult diseases, *e.g.* the metabolic syndrome and diabetes, is a high priority area in future research of LV. Two independent methods have been used to show the presence of LV in human IUFD cases.

Knowledge and evaluation of *ante*- and *post mortem* aspects of perinatal death is required to reduce and prevent these unexplained sudden deaths. We have focused on an infectious disorder which clearly needs further improvements of diagnostic and therapeutic tools. This requires early screening procedures and vaccination, in addition, the education of the medical community as well as the general public. The complexity of infectious disorders requires collaboration between several fields of medicine. Obstetrics is a discipline in which a multidisciplinary approach is already essential. Still this is a big challenge.

"Dubitando ad veritatem venimus".

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"Aristotle"

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