Summary

Background: Sudden infant death syndrome (SIDS) is defined as sudden unexpected death of an infant that remains unexplained after thorough post-mortem examination, investigation of the scene of death and case history. The autopsy findings and the physiological characteristics of these infants suggest a possible role of insufficient cardiorespiratory control and arousal mechanisms. The etiology is probably multifactorial based on a genetic predisposition combined with environmental factors. Several candidate genes have been studied, e.g. those involved in serotonin transport, autonomic nervous system embryology, inflammation, energy production, nicotine and glucose metabolism. A small number of cases may be caused by monogenic diseases that can lead to sudden death and leave no characteristic autopsy findings and thus imitate SIDS. Fatty acid beta-oxidation disorders (FAOD) have been associated with SIDS since 1976 and it is nowadays estimated that they may be responsible for about 1% of SIDS cases. Congenital long QT syndrome, a cardiac channelopathy, that may cause a fatal arrhythmia was a logical candidate for SIDS and indeed it was found out that about 9,5% of SIDS cases carry a mutation or a function changing variant in one of seven cardiac ion channel genes. We assumed that the severe salt wasting form of congenital adrenal hyperplasia (SW CAH) may lead to acute adrenal crisis with fatal consequences and imitate SIDS as well.

Aim: The aim of the study was to prove that the salt-wasting form of CAH may lead to sudden unexpected death of an infant without prior typical symptoms and thus is responsible for some cases of unexpleined deaths in infancy.
1a. We wanted to find out the prevalence of unrecognized CAH cases among suddenly deceased infants and hence support the introduction of neonatal screening for CAH.
1b. We wanted to find out the prevalence of FAOD among the same infants.

In addition, as we used newborn screening cards that have been stored for long periods of time, we had to perform studies that would allow us to reduce the effect of storage time and other factors on the level of acylcarnitines that are typically elevated in FAOD and amino acids that signify possible aminoacidopathies.
2. Hence, we tested long-term stability of analytes detected by tandem mass spectrometry in dried blood spots after prolonged storage.
3. We also investigated the influence of hemtocrit and position of the punch in the blood spot on analyte concentration.

Methods: 1. Newborn screening cards of infants who were born between 1989 and 2001 in the Czech Republic and Austria and who died between 7 days and 12 months of life without known cause were retrieved from neonatal screening archives in Prague and Vienna (257 samples altogether).

1a. Detection of CAH

The samples were analyzed by a solid phase fluoroimmunoassay (DELFIA) to determine the concentration of 17- alfa-hydroxyprogesterone (17-OHP). All samples with 17-OHP above the cut-off value were subsequently analyzed by gene-specific PCR and ligase-mediated mutation detection for detection of the nine most prevalent CAH-causing mutations of the *CYP21* gene at the Children's Hospital, Zürich, Switzerland.

1b. Detection of FAOD

In the second part of our experiment the retrieved samples were analyzed by tandem mass spectrometry (MS/MS). One sample was flagged as suspected of FAOD. The diagnosis of long chain hydroxyacyl CoA dehydrogenase deficiency (LCHAD/TFP) was subsequently

confirmed by mutation analysis performed in Vienna, which included PCR and restriction enzyme analysis.

2. Long-term stability of analytes in dried blood spots after prolonged storage

60 randomly chosen screening filter cards of children born in January of each of the following years 1989, 1991, 1993, 1995, 1997, 1999, 2000, 2001, 2002, 2003, 2004 (total n=660) were retrieved from the archives in the Czech Republic. The level of acylcarnitines and amino acids were determined in each sample by the same tandem mass spectrometer. The effect of storage time on the concentration of each substance was assessed by linear regression and yearly declines were estimated.

3. Influence of hematocrit and position in the blood spot on analyte concentration

Heparinised blood (50 ml) from a healthy adult was diluted so that samples with defined hematocrit levels (20%, 30%, 40%, 50%, 60%) were obtained. Forty dried blood spots were made for each hematocrit level. A central and a peripheral 3 mm disks were analyzed for amino acids, acylcarnitines and guanidine-acetate by MS/MS. The effect of hematocrit level and location were assessed by analysis of variance (ANOVA).

Results: 1a. Prevalence of CAH among sudden infant deaths

The diagnosis of CAH was confirmed in three out of 242 samples. Their newborn 17-OHP levels and *CYP21* genotypes were 706 nmol/l and del/conv//del/conv, 53 and I2//I2 and 811 nmol/l and I2//Q318X, respectively.

1b. Prevalence of FAOD among sudden infant deaths

In one out of 179 analyzed samples the diagnosis of FAOD was established retrospectively. The child was a homozygote for the most common mutation G1528C in the *HADHA* gene, which causes LCHAD/TFP deficiency.

2. Stability of analytes in dried blood spots after prolonged storage

Concentrations of free carnitine increased by about 7.6 % per year during the first five years of storage and decreased by about 1.4 % per year thereafter. Alanine, arginine, leucine, methionine and phenylalanine decreased by 6.5 %, 3.3 %, 3.1 %, 7.3 % and 5.7 % per year, respectively. Acetylcarnitine, propionylcarnitine, citrulline, glycine and ornithine decreased by 18.5 %, 27.4 %, 8.1 %, 14.7 % and 16.3 % per year during the first five years, respectively. Thereafter the decline was more gradual. Tyrosine decreased by 1.7 % per year during the first five years and 7.9 % per year thereafter. Valine was stable throughout the time period tested.

3. Influence of hematocrit and position in the blood spot on analyte concentration

Levels of most AA and GAA increased significantly with increasing hematocrit ($p < 0.001$), while the effect of hematocrit on some AA was less pronounced. Total AC, free carnitine, some long, medium and short chain AC correlated positively with hematocrit levels ($p < 0.001$). In samples with low hematocrit, levels of most AA and free carnitine were higher in the peripheral than in the central disk ($p < 0.0001$).

Conclusions: We confirmed that CAH due to *CYP21* gene defects may cause sudden unexpected death without previous typical symptoms and hence may be misdiagnosed as SIDS. In addition, we found out that the I2 mutation that is typically associated with the less

severe simple virilizing form of CAH may under certain circumstances lead to fatal outcome. We conclude that neonatal screening for CAH would have prevented these deaths. We found only one case of FAOD out of 179 sudden infant death cases, which corresponds to other studies. We also identified mathematical models that describe concentration changes of diagnostic analytes after prolonged storage and may be used to correct for concentration loss in retrospective studies.