1. Introduction

1.1 General background

Infancy, defined as the first year of life, is characterized by an extremely rapid growth rate along with the highest nutritional demands. During this period enormous changes in infants' diet take place, expanding from entirely milk-based to one including a variety of different foods and drinks. Optimal nutrition during this time of life has been shown as essential for best possible growth and development and risk of disease later in life(Berenson, 2002; Caballero, 2001; Guo *et al.*, 2002; The National Board of Health, 2005). Under-nutrition *in utero* has also been shown to cause permanent changes in the body's structure, function and metabolism, that may lead to disease in adulthood i.e. coronary heart disease, Type 2 diabetes and hypertension(Barker *et al.*, 1989; Barker, 2004).

Infants are a vulnerable group with no influence or choice on the composition of the diet why parents have great responsibility with regard to infant feeding. The majority of parents rely on official recommendations regarding infant and young child feeding, an area in constant focus aimed at recommending parents the best known diet for their children.However, accurate assessment of food intake in infants and young children is an essential prerequisite fordeveloping these recommendations and for conducting research on associations and cause-effect relationships between diet and health.

1.2 Dietary assessment among infants and young children

When measuring dietary intake among infants and young children different methods are available, however, the choice of method depends on the objective of the study. If the investigator is interested in patterns of food use and assessment of usual intake of foods during longer, less precisely defined periods of time, a food frequency questionnaire (FFQ) can be used. The usual frequency of consumption of different foods is reported retrospectively. Advantages of the FFQ are that it imposes less burden on the respondents (parents) compared to most other dietary assessment methods and is applicable in larger epidemiological studies. The data from a FFQ may be used to rank subjects into categories of different intakes of certain foods and compare these with the prevalence of a disease for example. Limitations of using a FFQ include lower accuracy than other methods, normally no achievement of quantitative data on food or nutrient intakes and the amount of work required for questionnaire development(Gibson, 1990). Some FFQs, known as semi quantitative, include portion size estimates where respondents are asked to indicate the frequency of consumption of specific quantities of foods or to assess their usual portion size based on a specified measure. The Norwegian national survey selected this method to provide information on dietary habits of infants and young children(Lande *et al.*, 2003; Lande *et al.*, 2004c; Lande *et al.*, 2005). The method has furthermore been used in studies in Australia, US and Denmark among this age group(Conn *et al.*, 2009a; Newby *et al.*, 2003; Nielsen *et al.*, 1998).

If the study aims at measuring the quantity of the individual foods consumed over a shorter period of time, recall or record methods are used. In the twenty-four-hour recall method parents of the infants and young children are asked by trained personnel to recall their child's exact food intake during the previous twenty-four-hour period or preceding day. The interviewer will record descriptions of all foods and beverages consumed, sometimes including cooking methods and brand names. Quantities of foods and beverages are usually estimated in household measures(Gibson, 1990). Advantages of the twenty-four-hour recall are that the method is quick, relatively inexpensive and the respondent burden is low. Furthermore it is useful for assessing average usual intakes of large populations. Limitations include that it relies on memory and single twenty-four-hour recall is likely to omit infrequently consumed foods (Gibson, 1990). The twenty-four-hour recall method has been used in large representative national samples of US infants and young children (0-47 mo) (Butte *et al.*, 2010; Devaney *et al.*, 2004).

Food records can either be estimated or weighed, where respondents record in detail all consumed foods at time of consumption and the recording continues for a specified number of days. The number of days included in records varies. It has been argued that 5 days or less of registration for infants and young children is sufficient(Black *et al.*, 1983; Erkkola *et al.*, 2011; Lanigan *et al.*, 2004),whereas others argue that 7 or 8 days are needed(Livingstone *et al.*, 2000b; Miller *et al.*, 1991). For estimated food records, portion sizes can be estimated from household measures and/or photographs. In the weighed food record (WFR) method the parents are asked to weigh and record all foods and beverages consumed for a specified time period. Details of cooking methods and brand names may also be collected. Advantages of the food records include the ability to measure actual or usual intakes of individuals, they may be open-ended and do not rely on memory or the restriction to a limited number of food items. Limitations include that the respondents have to be literate and highly motivated since the respondent burden is large. Furthermore, a possibility of underreporting the intake or eating differently during the recording period exists. The WFR may be more costly and time consuming to implement compared to the estimated records, because estimated food records can permit scanning during the data handling process, reducing the researchers' workload considerably compared to the workload in datahandling a WFR. Estimated food records have been used in several studies including two studies among infants in the UK(Mills *et al.*, 1992; Noble *et al.*, 2001c), among infants in Iceland (Atladottir *et al.*, 2000c), and more recently among Finnish children (1-6 yrs)(Kyttala *et al.*, 2010b).

The WFR has long been recognized as the most precise method available for estimating usual intakes (Gibson, 1990) and has been used in a number of studies with infants and young children in Europe, US and New Zealand (Alexy *et al.*, 1999; Heinig *et al.*, 1993d; Michaelsen, 1997a; Schiess *et al.*, 2010; Szymlek-Gay *et al.*, 2009; Atladottir *et al.*, 2000b).

An overall better understanding of the advantages and limitations of the different methods, has led researchers to creative blending of different methods, utilizing the strengths of each method(Thompson *et al.*, 2011). In the Dutch national food consumption survey, parents of young children (2-6 yrs) kept dietary records for two non-consecutive days after which dieticians entered the data from the records into a twenty-four-hour recall computer software (Ocké *et al.*, 2008). Similar record-assisted twenty-four-hour recalls have been developed for older children(8 yrs) in the US (Lytle *et al.*, 1993).

Obtaining accurate measurements of food, energy and nutrient intake in infants and young children can be very challenging, but also includes some advantages. As the parents normally control the dietary intake of infants and young children and that this age-group primarily eats at home, this provides potential for an accurate recording. Among challenges faced, one is when other caregivers than the parents are involved in the recording, e.g. at day-care, these caregivers may approach the task of recording with varying levels of motivation and interest. Other challenges may include conscious or unconscious modification of usual dietary habits in an attempt to present a more wholesome picture and the problems of estimating the amount being eaten as some might be wasted, perhaps not only on the plate but around and on the child. Furthermore, it may be very difficult to estimate the amount of breast milk consumed for infants being breastfed and may result in rather crude estimates. Lastly, challenges after data collection are present, i.e. determining correct portion sizes and recipes and utilizing the optimal food database. These reasons and others make it difficult to obtain accurate assessment of dietary intake in infants and young children and place high requirements on the method being used.

Validation studies provide an estimate of the validity of the method in focus. Assessing the validity of a method refers to the degree to which the method actually measures the true intake the method was designed to measure (Livingstone *et al.*, 2003b), an essential matter for a study. Several studies of estimated food records in children and adolescents have shown that a major problem is an average underestimation of food intake by up to 35% (Bandini *et al.*, 1997; Champagne *et al.*, 1998; Singh *et al.*, 2009). However, only little research on the validation of dietary intake methodology in infants and young children exist(Burrows *et al.*, 2010b; Davies *et al.*, 1994d; Lanigan *et al.*, 2001a).

1.2.1 Doubly labelled water (DLW) technique

1.2.1.1 Background

The DLW technique is a technique for estimating total energy expenditure (TEE) by use of stable isotopes. TEE measured by the DLW technique is considered to be the gold standard reference method for validation of measurements of energy intake (Ainslie *et al.*, 2003).The basic assumption for the method is that it is measured under energy balance (energy intake (EI) =TEE), which may impose challenges for certain age groups.

DLW is a mixture of stable isotope labeled waters (^2H_2O and$H_2{}^{18}O$), including the stable isotopes deuterium (^2H) and oxygen (^{18}O).Hydrogen consists of a nucleus with one proton and one electron. This isotope is also called protium (^1H). In deuterium (^2H), the heavier stable isotope of hydrogen, the nucleus also contains one neutron. The nucleus of the most common isotope of oxygen, oxygen-16(^{16}O), contains 8 protons and 8 neutrons. The heavier stable isotope used in the DLW technique,oxygen-18 (^{18}O),has 8 protons and 10 neutrons in its nucleus(IAEA, 2009).

The stable isotopes occur naturally, but only 0.015 % of all hydrogen is deuterium, while approximately 0.20 % of all oxygen is oxygen-18 (IAEA, 2009). This means that an infant weighing 10 kg with approximately 6 kg of body water (Butte *et al.*, 2000a) contains around 12 g ^{18}O, but less than 1 g ^2H. Consequently, mammalian cells are accustomed to molecules containing ^2H and ^{18}O at natural abundance(IAEA, 2009).

Deuterium (^2H) may, because of the relatively large mass difference from protium (^1H), induce adverse effects at the cellular or whole organism level. High concentrations of deuterium can theoretically cause a depression of tissue metabolism due to lower reaction rates of deuterium-labelled compounds *in vivo*. However, harmful effects in mammals have not been detected in concentrations in body water below 15 % and the deuterium doses needed to assess TEE will give a maximum concentration in body water of about 0.12 %(IAEA, 2009). In summary, there is no evidence regarding disadvantageous long-term effects of deuterium in doses used for clinical study(Koletzko *et al.*, 1997b). In contrast to deuterium, the heavier stable isotope, oxygen-18 differs relatively little in mass from the predominant form of oxygen and has not shown any adverse effects even at highest enrichments(IAEA, 2009; Koletzko *et al.*, 1997a). Consequently, deuterium and oxygen-18 are considered safe for use in humans across the lifespan.

1.2.1.2 Theory

The DLW technique is based on the administration of a DLW(^2H_2O and$H_2{}^{18}O$) dose and the assessment of the elimination rates from a body fluid such as urine. The urine should ideally be collected for a period of time equivalent to 1-3 half-lives for isotope clearance. For infants, having a higher water turn-over rate than adults, sampling for one week is often appropriate (IAEA, 2009). As ^2H is eliminated exclusively as water, whereas ^{18}O is eliminated from the body as carbon dioxide and water, the carbon dioxide production rate can be estimated from the difference between the two elimination rates. Oxygen consumption is calculated using an estimate of the respiratory quotient (RQ) and TEE can be calculated (IAEA, 2009). RQ can either be derived from information on the subjects' dietary intake or average data from population surveys (Roberts *et al.*, 2005a).

The basic assumption for the DLW technique where EI equals TEE is not valid in children due to growth. Hence, to compare EI with TEE,a factor equivalent to the energy cost of growth, has to be added TEE to derive metabolisable energy intake (ME_{DLW})(Davies *et al.*, 1994c).

Despite that the DLW technique is considered to be the gold standard reference method for validation of measurements of energy intake, the technique is still rarely used due to the high costs and significant technical skills and facilities required for the analysis(Burrows *et al.*, 2010a; Westerterp, 1999b).

1.3 Types of study

When investigating relationships between nutrition and health, a number of research methods are available. *In vitro* and animal models are primarily used to generate knowledge about mechanisms of nutrient action and dose-effect relationships, whereas experimental and observational studies, in humans, constitute the basis for diet and health relationships (Nordic Council of Ministers, 2004). In experimental studies, including randomized controlled studies, the researcher manipulates exposures by assigning the subjects to groups either receiving an intervention or exposure or not. The subjects are subsequently followed under controlled conditions. In observational studies, including case-control studies, cohort studies and cross-sectional studies, researchers measure the exposure or treatment and observe outcomes as they occur. As randomized, controlled studies are difficult to apply in large groups for a longer period of time, observational studies have become important for evaluating the relationships between nutrition and health (Nordic Council of Ministers, 2004). However, an important limitation for the observational studies are that they are normally limited to explore associations and can only rarely establish a causal relation (Nelson *et al.*, 2004; Stender *et al.*, 2010).

1.4 Infant diet

At 9 months of age all infants in Denmark should, according to the official recommendations, be at the last stage of weaning where they are introduced to family foods (The National Board of Health, 2005). The official Danish recommendations recommend exclusive breastfeeding up until 6 months, however, complementary foods may be introduced from 4 months of age to support optimal growth and development (The National Board of Health, 2005). The timing of the first introduction of complementary foods has shown to be an important factor for subsequent health. Large cohort studies have shown a protective effect of later introduction to complementary foods in relation to infancy weight gain (Baker *et al.*, 2004c), adult overweight (Schack-Nielsen *et al.*, 2010) and respiratory illness during childhood (Wilson *et al.*, 1998).A longer duration of breastfeeding is associated with a delayed introduction of complementary foods (Baker *et al.*, 2004b; Burdette *et al.*, 2006; Kramer, 1981)and results from a large cohort study showed an interaction between breastfeeding and introduction of complementary foods, suggesting that the combination of short periods of breastfeeding and early introduction of complementary foods may contribute to increased infancy weight gain (Baker *et al.*, 2004a).

Several studies have reported differences in food intake between infants being partly breastfed and those completely weaned in the second half of infancy, where foods and drinks become an increasing part of the diet (Heinig *et al.*, 1993c; Lande *et al.*, 2004b; Michaelsen, 1997b; Noble *et al.*, 2001b; North *et al.*, 2010). Little is known about the link between breastfeeding and dietary habits in late infancy, but it is reasonable to speculate that infants introduced late to complementary foods will be delayed in their progression towards spoon-feeding and thereby family foods. Information on this is interesting for investigation concerning breastfeeding and later health outcomes and for counselling mothers in healthy infant feeding practices.

1.5 Iron

The demand for nutrients during infancy is extremely high because of the high growth rate, and not all requirements can be met from a diet without fortified products. Iron deficiency[1]is one of the most common nutrient problems in infancy, both in affluent as well as in developing countries, and may progress into iron deficiency anemia[2]. In developing countries the prevalence of anemia, as an indicator of iron deficiency anemia is high, amounting to almost 70 % among children less than 5 years of age (World Health Organization *et al.*, 2008). In Europe anemia is less prevalent, but still more than 20 % of children 0-5 years of age are estimated to be anemic (World Health Organization*et al.*, 2008).Although iron deficiency is not the only, it is by far the primary cause of anemia (Benoist *et al.*, 2001).

[1] Defined as depleted iron stores (serum ferritin concentrations < 12 µg/L) (World Health Organization, 2001; World Health Organization *et al.*, 2004).

[2]Depleted iron stores in combination with anemia, defined as haemoglobin concentrations in blood beneath the following cut-off values: Children (0.5-4.99 y): 110 g/L, children (5.0-11.99 y) 115 g/L, children (12.0-14.99) and non-pregnant women: 120 g/L, pregnant women: 110 g/L and men (≥15.0 y):130 g/L (World Health Organization *et al.*, 2008)

The consequences of iron deficiency are extensive and increase in severity with increased depletion. The most serious consequence of iron deficiency is anemia, a condition which may be associated to delayed mental and motor development in children (Booth *et al.*, 1997; Grantham-McGregor *et al.*, 2001).It is uncertain whether such consequences are reversible (Aggett *et al.*, 2002).Other consequences of iron deficiency are decreased growth of infants and young children, and decreased physical capacity and reduced immunity for all age groups (World Health Organization, 2001).

The development of iron deficiency is characterized by changes in the iron stores of the body. In the first stage, iron stores become depleted with still enough iron to meet the need for red blood cell production. In the final stage iron stores become exhausted, the amount of iron in the circulation is very low, red blood cell production is drastically reduced and iron deficiency anemia develops (Fairweather-Tait, 1993).

Complementary feeding diets include a broad range of foods containing iron, where fish, meat and chicken are especially good sources of iron due to the high content of haem-iron, which is easily absorbed and promotes the absorption of non-haem iron from other foods (Cook *et al.*, 1976; Hallberg *et al.*, 1979). In contrast, several components in the diet, like phytic acid and calcium, may reduce the amount of bioavailable iron (Hallberg *et al.*, 1989; Hallberg *et al.*, 1993).

In contrary to most countries, Denmark recommends that infants not receiving at least 400 ml iron-fortified formula and/or follow-on formula per day receive iron supplements (8 mg iron/day) from the age of 6 to 12 months (The National Board of Health, 2010). Iron supplements, most common in the form of iron drops, are easily distributed, have long shelf-life and are less expensive than fortifying foods with iron. However, risks and benefits of iron supplementation are likely to vary between populations and whereas evidence for the efficacy of iron supplementation for iron-deficient infants and young children exists in developing countries (Iannotti *et al.*, 2006), several randomized controlled trials, in both affluent and developing countries, have found significant adverse effects in infants with normal iron status (Dewey *et al.*, 2002; Idjradinata *et al.*, 1994; Majumdar *et al.*, 2003; Sachdev *et al.*, 2006).

The benefits of following the Danish iron supplementation recommendation on iron status in Danish infants have not been thoroughly examined. However, we presume that the iron status is improved in those infants following the iron supplementation recommendation compared to infants not following the recommendation.