DEPARTMENT OF BIOSCENCES AND NUTRITION
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Polyamines in Foods and Human Milk

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To my great parents with all respect

To my wonderful sisters and devoted brothers

ABSTRACT

Background: Knowing the levels of polyamines, putrescine, spermidine and spermine in foods and human breast milk, and the contribution of daily food choice to polyamine intake and its effect on the levels in breast milk is of interest, due to the association of these bioactive amines to health and disease. There is a lack of relevant information on the content of polyamines in the Swedish Food Database. Polyamines in human milk vary between lactating mothers. In this thesis, a polyamine database was developed through literature review and laboratory analysis of polyamines in Swedish dairy products. We also aimed to estimate polyamine intake among adolescents and lactating mother in order to compare the intake with Swedish Nutrition Recommendations Objectified (SNO) and associate it with the levels in breast milk, respectively. The effect of a weight reduction intervention program on the levels of polyamines in milk from obese lactating mothers was also investigated.

Methods: Polyamine contents of foods were collected and polyamine data were inserted into the Swedish food database after an extensive literature search of databases and laboratory analysis of Swedish dairy products. Polyamine intake was calculated using Dietist XP after obtaining 7-day food records from 93 adolescents. Human milk samples were collected one week after delivery from mothers with normal BMI delivering prematurely after 24-36 wks of gestation (n=40) and after full term delivery (n=12). Milk was also collected after full term delivery at days 3 and 10 and at 1 and 2 months in normal weight (n=20), obese (n=20) and obese mothers who had participated in a weight reduction program during pregnancy (OI, n= 10). Food records for 3 days were obtained covering the sampling day. Polyamine levels in all samples were analyzed using high performance liquid chromatography (HPLC).

Results: Fruits and cheese were identified as the best sources of putrescine, while vegetables and meat products were found to be rich in spermidine and spermine, respectively. The adolescents' polyamine intake was 316 ± 170 µmol/day, while the calculated contribution from the ideal diet SNO was considerably higher with an average polyamine intake of 541 µmol/day. Polyamine concentrations were higher in preterm than in full term milk and higher in human milk than in the corresponding formulas. Dietary intake of polyamines was associated with their content in human milk (putrescine $r = 0.72$, ($p < 0.0001$); spermidine $r = 0.76$ ($p < 0.0001$); and spermine $r = 0.53$ ($p = 0.003$)). Total polyamine concentrations were higher in milk from obese mothers with intervention (703.9 ± 31 nmol/dl at 3 days, 767.5 ± 31 nmol/dl at 1 month and 727.2 ± 28.2 nmol/dl at 2 months) than the obese control mothers (571.2 ± 25.3 nmol/dl at 3 days, 603.2 ± 24.2 nmol/dl and 567.6 ± 22.3 nmol/dl at 1 month and 2 months, respectively), ($p < 0.01$).

Conclusions: The database provides information for other researchers in their quest for information regarding polyamine intake from foods. The average daily total polyamine intake was low in comparison with an intake estimated from healthy diet recommendations. None of the formulas reached the total concentration in corresponding breast milk. The strong correlation between breast milk content and mother´s intake, and the higher concentrations in milk from obese women after general dietary intervention at all lactation times compared with that from both normal weight and obese women, suggest that dietary advice can improve the contents of breast milk.

Keywords: Putrescine; spermidine; spermine; foods; polyamine intake; breast milk, formulas.

LIST OF PUBLICATIONS

I. Ali MA, Poortvliet E, Strömberg R, Yngve A. Polyamines in foods: development of a food database. *Food Nutrition Research 2011, 55: 5572. DOI: 10.3402/fnr.v55i0.5572*

II. Ali MA, Poortvliet E, Strömberg R, Yngve A. Polyamines: total daily intake in adolescents compared to the intake estimated from the Swedish Nutrition Recommendations Objectified (SNO). *Food Nutrition Research 2011, 55: 5455 - DOI: 10.3402/fnr.v55i0.55455*

III. Ali MA, Strandvik B, Sabel KG, Kilander CP, Strömberg R, Yngve A. Polyamine levels are associated with mothers' dietary intake and higher in preterm than full term human milk and formulas. *Submitted.*

IV. Ali MA, Strandvik B, Kilander CP, Yngve A. Polyamines in breast milk from obese and normal weight mothers with and without a weight reduction program. *Manuscript.*

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List of abbreviations

BMI	Body Mass Index
CVD	Cardiovascular diseases
ODC	Ornithine decarboxylase
PAO	Polyamine oxidase
SPM	Spermine
SNO	Swedish Nutrition Recommendations Objectified
SPD	Spermidine
WHO	World Health Organization

Preface

Three years before I got my scholarship that changed my life, not just scientifically, I had started my first official job as nutritionist in one of the biggest children hospitals in my country. I was surrounded by professional medical doctors and surgeons, nurses, and technicians, who all knew exactly what their tasks were. I, on the other hand, was keen to understand and deal with known and new, simple and complicated nutritional issues. My colleagues and I were trying to prove that nutrition is an important and a core element in the clinical setting. We tried by being present not only for the patients and their mothers in all wards and the nutrition clinic, but even in the hospital catering. However, I always kept asking myself the question: *there is still something missing here, what is that?*

Working with this PhD project came to answer my question unintentionally. I came to open my mind to things that I had never learned before about nutrition which is not just science. Nutrition is an art and to make it work, the tool has to be there. *I came to realize that I was missing the tool*.

Preparing for this thesis made me use, as much as I can, useful and fantastic means that can lead to a significant outcome and a more developed understanding of how to solve problems related to either estimating dietary intake or detecting amounts of different compounds in foods. I also got to know that nutrition is not only telling people what they should and should not eat, but also involving them in practical interventions that can make changes for them and future generations. It is about making them feel that what we and they need to see is not the medicine or the vitamin pill in the drawer, but it is the fresh and healthy food in the kitchen; what they need to consider is not the quality of the formulas they buy for their children, but the type of food the lactating mothers should eat. I was not only missing the research that involves those mothers and their children in healthy patterns, but also the knowledge to make that happen.

These years of PhD training provided me with a big chance to understand the concept of research and a good opportunity to work with scientists who offered me the tool and taught me how to make things work.

I have come from a society that some part of it is still trapped by the myth "*to be healthy and strong is to eat too much*!" I hope that will change one day.

Mohamed Atiya Ali

Huddinge, August 2011

1 INTRODUCTION

What we eat can influence and determine our health and disease during growth and development, and later in life. Throughout the neonatal period and infancy, breast milk is the golden standard for protective nutrients and bioactive nutrients that have a protective role against many diseases. A healthy, balanced diet is one of the essential elements in disease prevention. The nutritional significance of a healthy food and human milk and their important role in promoting health and prevention from diseases are believed to be not only a reflection of the nutrients they contain, but also due to the bioactive compounds they provide.

Putrescine, spermidine and spermine are small aliphatic polycations that belong to a group of biologically active amines and found in variable amounts in almost all kinds of foods (1) (figure 1). Due to their specific biological roles they are now classified as a separate and peculiar group known as polyamines, and have received special interest in the fields of nutrition and food research (2). In addition to their external dietary sources, polyamines are also ubiquitous in all cells and produced in the normal metabolism. Among many nutrients and non-nutrients in foods, recent studies have focused on the importance of polyamines because of the recent reports on the health benefits and biological significance of dietary polyamines (3). Even though polyamines are synthesized by almost all mammalian cells (4, 5), dietary sources are essential to maintain the body pool of polyamines and to regulate polyamine biosynthesis (6, 7). This thesis is an attempt to describe aspects of polyamines in foods and their intake among adolescents. It is a way of providing dietitians with information that can be needed when estimating polyamine intake. In addition, the thesis demonstrates how the level of polyamines in human breast milk can be influenced by the mother's diet during lactation and by means of a weight reduction intervention program during pregnancy and lactation.

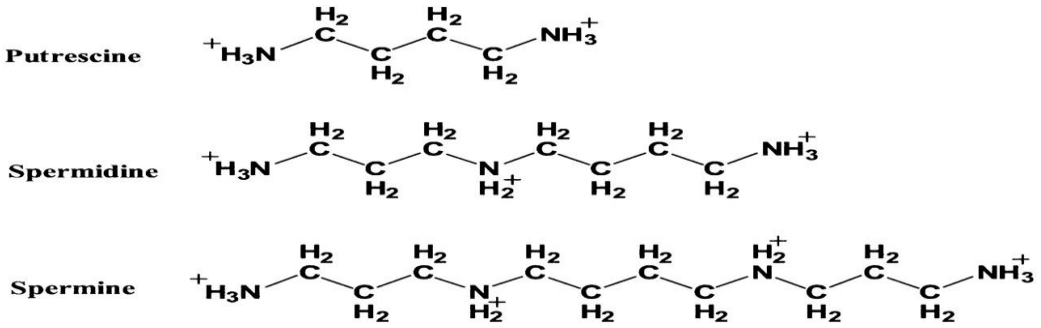


Figure 1. Chemical structures of putrescine, spermidine, and spermine (8)

1.1 SOURCES OF POLYAMINES

The source of polyamines can be either endogenous, through intracellular de novo synthesis, or exogenous, through dietary uptake and absorption from intestinal microflora. As shown in figure 2, in the cell, polyamines can be synthesized from ornithine by the action of the enzyme ornithine decarboxylase (ODC), to produce putrescine. Consequently, spermidine and spermine are then formed by the action of the enzymes spermidine synthase and spermine synthase, respectively. Another enzyme that is considered essential for formation of spermidine and spermine is S-adenosylmethionine decarboxylase (9).

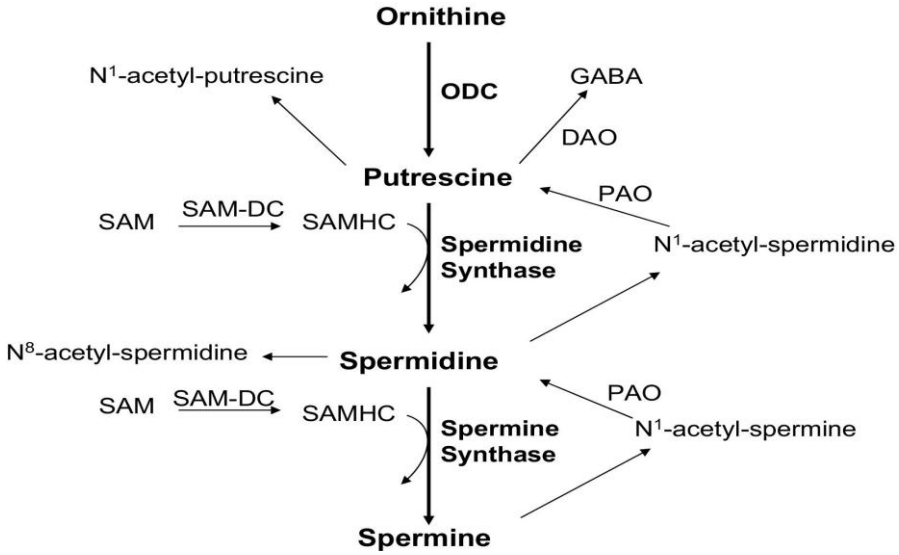


Figure 2. Intracellular polyamine synthesis. DAO, diamine oxidase; GABA, γ aminobutyric acid; PAO, polyamine-oxidase; SAM, S-adenosylmethionine; SAM-DC, S-adenosylmethionine decarboxylase; ODC, ornithine-decarboxylase; SAM-HC, S-adenosylmethionine homocysteamine (9).

The main exogenous source for polyamines is diet, particularly fruits, vegetables, cheese, and meat (4, 10). In addition, human milk also contains substantial amounts of polyamines (11). The other exogenous source is the gastrointestinal bacterial flora (12, 13).

1.2 POLYAMINE METABOLISM

Polyamines are absorbed in the duodenum and the first portion of the jejunum (9, 14). Even though polyamines are rapidly and completely absorbed, only small fractions (15-20%) of putrescine is recovered in blood (4, 14), , while 75 to 80 % of spermidine and spermine remained in their original form in the circulation (4).

Once polyamines reach the blood circulation, they are absorbed by several tissues such as liver, intestine and thymus (4, 9), where polyamine inter-conversion and synthesis takes place (figure 2). In addition to ornithine, the amino acid arginine is a precursor to the latter and therefore to polyamines (15), while the amino acid methionine is also involved in the metabolic pathway of polyamine synthesis, particularly the formation of spermidine and spermine (9). It is clearly established that diet is an important source to provide the polyamines required to maintain the normal metabolism (4, 16).

1.3 BIOLOGICAL SIGNIFICANCES OF POLYAMINES

Why are polyamines important?

The multiple functions of these molecules make them essential for life. There has been an increase of interest regarding the array of polyamine roles in maintaining cellular and body function. Their importance in cellular growth and proliferation has been established in humans and several animal species (17). The ability of polyamines to stabilize and regulate the DNA and RNA and interact with other components of the cell membrane is essential for maintaining cellular functions and protein synthesis (18-23). The involvement of polyamines in both cellular differentiation and regulation of inflammatory reaction makes them play an essential role in facilitating wound healing (24).

1.3.1 Polyamines as antioxidants

The protective roles of polyamines as antioxidant agents in several tissues have been reported in several studies (1, 25-27). Spermine has been shown to be the most efficient among polyamines in protection from DNA damage (28, 29). In vitamin E deficient rats, putrescine and spermidine have been shown to exert an antioxidant function in rat lungs exposed to oxidation, suggesting that polyamines may play an antioxidant role in compensating for vitamin E deficiency (30). Moreover, the activity of polyamines as antioxidants can be even stronger than that of antioxidant vitamins (26). The DNA and cell membrane, which are targets to reactive oxygen species, are also protected from oxidation by their high polyamine concentrations (31).

1.3.2 Anti-inflammatory properties of spermine and spermidine

In vivo and *in vitro* studies have shown that spermidine and spermine exert an anti-inflammatory effect by inhibiting the pro-inflammatory cytokines and decreasing the expression of leukocyte function-associated antigen-1, one of the molecules that are needed to trigger inflammation (32, 33). In addition, animal studies have shown that feeding mice with polyamine-rich diet increases their longevity by decreasing the incidence of age-associated diseases, such as glomerulosclerosis, which is an age associated condition linked to atherosclerosis (34, 35). This would reflect the importance of polyamine intake from foods.

1.3.3 The effect of polyamines on the gastrointestinal tract

The significant role of these biogenic amines in the growth and development of the gastrointestinal tract during rapid physiological growth seems to be important not only at this stage. Polyamines are also necessary for the maintenance of the normal growth and general function of the adult digestive system (36). This role of polyamines has been observed in animal studies either by introducing these substances in the lumen or by reducing their supply. Infusion of putrescine in the lumen of fasted rats produces a significant increase in mucosal protein synthesis and exerts a mucosal growth (37). In addition, intragastric administration of polyamines, particularly spermine, induces the healing of gastric mucosal ulcers and lesions induced by either stress or acidified ethanol in rats (27, 38).

1.3.4 Polyamines and diabetes

Another area in which the biological roles of polyamines are receiving great attention is diabetes. This is not only due to their indirect possible role in prevention of hyperglycemia, but also because of their significant antiglycation effect to prevent from diabetes complications. Polyamines have been shown to exert an impact on insulin production and secretion. It has been reported that the role of spermine and spermidine in insulin production arises from their stimulation to insulin release and their involvement in mediating the rapid islet cell proliferation (39). The study has shown that in pancreatic cells isolated from obese and hyperglycemic mice, polyamine depletion decreased the islets cell proliferation as well as the insulin release from these cells. Further, the role of glycation in the genesis of diabetic complications has triggered the needs for antiglycation agents that can attenuate the development of a series of diabetic vascular complications (15, 40, 41). An *in vitro study* has shown that polyamines exhibit an antiglycation effect by inhibiting the reaction of sugar with protein and thereby acting as natural antiglycation agents at physiological levels (42).

1.4 POLYAMINES IN FOODS

Although cells can synthesize polyamines, the diet seems to be an essential source that provides both polyamines and amino acids to maintain the cellular synthetic capacity and requirements (6). Dietary polyamines play an important role in childhood, adolescents and in elderly (43). The importance of dietary polyamines and their contents in foods have been receiving major attention, especially since the first report on polyamine contents in different foods which was published by Bardocz et al. (44) in the beginning of the nineties.

A normal adult diet provides several micromoles of polyamines per day. The polyamine distribution in foods includes almost all foods and food groups. Among dairy products, cheeses, mainly matured ones, are rich in polyamines, while milk and yogurt contain lower concentrations. Fermented food products such as sauerkraut, soybean and some sausages also contain high concentrations of polyamines due to the bacterial fermentation process that leads to the simple decarboxylation of some amino acids by the decarboxylases of some microorganisms (45, 46).

Polyamines are found in food in two forms: free and conjugated. The conjugate form is mostly found in plant foods, bound covalently to a partner molecule such as phenolic compounds or membrane phospholipids (2, 36, 43). In animal tissues, polyamines are supposed to be binding with proteins; however, it has not been confirmed yet (36). Until now, most of the published articles did not differentiate between these two different forms of polyamines (36, 43).

The distribution of the different polyamines in foods varies according to the type of food; meat and meat products are rich in spermine, while foods of plant origin contain mostly putrescine and spermidine.

Studies that reported the contents of polyamines in different foods showed considerable variations. These were not only between the different studies, but even in polyamine levels between the samples of the same type of food. Storage of foods, seasonal variations, food processing and cooking are all possible factors that may lead to this variation (47, 48). Putrescine can increase up to 8 times its original concentration in food products that are stored and exposed to microorganisms. Spermine and spermidine can be lost during storage of food as well as by changing in the storage temperature which lead to certain enzymatic degradation reactions (43). It has been determined that a three-month storage of chicken can lead to shrinkage reduction of 70 and 80% of the original content for spermidine and spermine, respectively (49).

1.5 THE SWEDISH FOOD DATABASE

The Swedish Food Database provides regularly updated information on the nutritional composition for more than 2000 foods and dishes, mostly Swedish representative foods (50). This Database as such enables the National Food Administration (NFA) to calculate energy and nutrient intakes from dietary surveys performed at the NFA. The database provides figures on more than 50 nutrients for each type of food. These are presented in terms of amounts per grams or portions of foods. In addition, some information on analytical methods, calculations and factors used in the calculations, are also available in the Swedish database.

1.6 THE SWEDISH NUTRIENT RECOMMENDATION TRASNLATED TO FOODS

The Swedish National Food Administration have translated their nutrient recommendations to foods in the resource "Swedish Nutrient Recommendations Objectified" (SNO) and formulated food lists contributing to 9.1 MJ and 11.5 MJ for healthy females and males with little to moderate physical activity levels, which provide the recommended levels of nutrients (51).

A range of foodstuffs were chosen from the Swedish food database. These were considered representative of Swedish eating habits and of what might comprise a balanced varied diet that. The foods selected were also nutritionally representative of their food group.

The SNO report provides a food list compiled with the average amounts to be consumed per day and per week. This list is made for different food groups in order to achieve the two energy levels. SNO can also be an important tool, e.g. for evaluating diets or/and for demonstration of what a healthy diet can look like. Therefore, considering SNO as a tool in estimating the degree of polyamine intake based on general dietary advice is of great interest.

1.7 INTAKE AND IMPORTANCE OF DIETARY POLYAMINES

Diet as an external source provides larger quantities of polyamines than the endogenous biosynthesis. It has been reported that only about 1-2 nmol of putrescine is produced per hour per gram of tissue in the most active organs (6). Thus, the dietary intake of polyamines is essential for maintaining optimal health.

Even though the requirements of polyamines are high during the stage of intense growth especially for children and adolescents, polyamine intake from diet is considered necessary for all age groups. In addition, polyamine intake from diet is presumably important for elderly. Animal studies have shown that the levels of spermidine and spermine decreased with age in rat tissues from liver, thymus, spleen, brain, kidney and muscles (52). Similarly, another study on mice has reported that the contents of spermidine decreased significantly with aging in the thymus, spleen, ovary,

liver, stomach, lung, kidney, heart, and muscles (53). The studies suggested the importance of dietary polyamines for maintaining the function of these organs. However, the recommended intake for polyamines is yet to be established.

The mean dietary intake of polyamines has been estimated in some countries like Japan (54), United States (55) and United Kingdom (4). The estimated daily polyamine intake in these studies varies between 250 to 550 µmol per day. In contrast, the total intake in southern Europe was even higher (700 µmol/day) (6). This difference in polyamine intake between regions has not only been associated with the differences in dietary patterns but also with the differences in the incidence of chronic diseases from which the Mediterranean diet is known to be protective (6). Recent studies suggest a possible contribution of increased intake of polyamines, which are abundant in the Mediterranean diet, to prevention from age associated diseases and prolonging human life (34, 56) . In addition, based on dietary information and data on polyamine concentrations in foods, it has been also concluded that a generally healthy eating pattern is significantly associated with higher intake of polyamines (3).

While the high intake of dietary polyamines is known to be protective, a diet deficient in polyamines can lead to undesired health outcomes. In mice or rats, polyamine deficiency caused hair loss and infertility in females (57), pancreatitis (58), and impairment of spatial learning (59). Further, in humans, spermine deficiency has been reported to be associated with neurotransmitter/circulatory problems, indicating the role of polyamines in brain development and cognitive function (60).

Despite the beneficial effect of dietary polyamines, it has been reported that a high intake of polyamines can induce proliferation of cancer cells in tumors that already exist (46). *In vivo* studies showed that the undesirable polyamine uptake by tumor cells is not only induced by endogenous polyamines, but also those from dietary sources released into the circulation (61). For instance, dietary polyamines were shown to induce carcinogenic growth in rat colon (62). Further, the effectiveness of the chemo-preventive agents against colon cancer was partly reduced by dietary polyamines (63, 64). Therefore, a polyamine reduced diet to control tumor growth has been used in some studies and at least one list of rich polyamine foods has been produced so it can be used in reducing polyamine intake in some risk group (64).

1.8 POLYAMINES IN HUMAN MILK AND FORMULA

Breast feeding is clearly the optimal source of nutrition for human infants. Besides the essential macro- and micronutrients, human milk contains a range of bioactive substances that modulate the newborn metabolism (5). Feeding breast milk compared with formula has been acknowledged to be associated with low blood pressure (65, 66), low incidence of obesity (67) and diabetes mellitus type 2 (68) later in life. This might be related to the high nutritional value and the various bioactive constituents of breast milk. Human milk and that from other mammalian species contain substantial amounts of putrescine, spermidine and spermine (69-71). The occurrence of polyamines in human and cow's milk was first detected by Sanguansermsri et al. (11). Since then, the interest in detecting polyamine concentrations in milk has been increasing. The levels of polyamines in infant artificial formulas were identified during the nineties; however the last time when data was reported on polyamine concentrations in formulas was in 1995 by Buts et al. (72). Compared with artificial powdered formula, polyamines in human milk have been reported to be found in higher concentrations (5, 72). This possibly reflects the importance of human milk polyamines in infant nutrition.

1.8.1 The significance of polyamines for newborn and during infancy

After the period of intrauterine life, the child is born with an immature digestive tract. The intestinal maturation occurs during the breastfeeding to weaning phase transition (71). During postnatal development, the intestinal epithelium undergoes marked structural and functional changes such as increase in mucus production, immunological adaptation to new microbial and nutritional antigens and digestive adaptation to new nutrients (43). The result of such changes is a functionally mature intestine containing the digestive enzymes that are necessary to cope with the diet of the adult. Several hormonal and nutritional factors from the milk are necessary to complete the intestinal epithelial maturation. It has been shown that polyamines play a significant role in regulating this biological process by promoting cellular differentiation and proliferation. In suckling rats and mice, oral administration of spermine and spermidine was associated with the maturation and proliferation of the intestinal mucosa (73) and increased significantly the weight and the length of the small intestine (74).

Further, an *in vitro* study has shown that when intestinal epithelial cells have been stimulated with different types of milk, the human milk, as a plentiful source of bioactive polyamines was more efficient in sustaining cellular growth than bovine milk and infant formula (75). This indicates the importance of breast milk over the formula feeding in the growth and development of the digestive tract.

It is generally accepted that the intestinal permeability to proteins is higher during the first 3 months of infancy than later in life (76, 77). During this period, antigenic molecules can cross the intestinal epithelium triggering allergic and immunological reactions (77, 78). Polyamines, particularly spermine and spermidine can prevent allergy by their previously mentioned role in postnatal intestinal maturation, and thereby decreasing this permeability to allergic particles from food. In addition, polyamines can increase the percentage of intra-epithelial lymphocytes, suggesting an important role of polyamines in maturation of intestinal immune system and regulation of its response to antigens (79). Therefore, a possible protective effect of breast milk against allergies, as described below, could be explained by its higher level of polyamines than the formula.

Epidemiological studies on children and lactating mothers have indicated that a high spermine and spermidine intake from breast milk during postnatal life may have protected children from food allergy by the age of 5 years (78, 80). They believed that allergic children had consumed human milk that contained significantly lower spermine and spermidine than that of non-allergic children.

Such observations point at the existence of variations in polyamine contents of human milk between mothers, and not only between human milk and formulas.

1.8.2 Factors that influence the content of polyamines in human milk

Although human milk contains substantial amounts of polyamines that are higher than formula, the concentrations in breast milk may depend on many factors. Besides the genetic and other environmental factors that can affect the human milk composition, the level of polyamines in human milk can be influenced by duration of lactation, and mother's diet and nutritional status (5, 81). Several studies on human milk reported variability in milk polyamines over the lactation period (11, 70, 82). In addition, different values in polyamine concentrations were observed between the studies (69,

70, 72) (Table 1). This difference has been explained by the possible effect of dietary intake and ethnicity (11). A study on the effect of polyamine and amino acids supplementation on rat milk demonstrated that diet could be very important to control milk composition of polyamines (81).

Table 1. Polyamine concentrations in human milk (nmol/dl)

Reference	Putrescine	Spermidine	Spermine	Total
Pollack et al	0-61	73-351	72-448	145-860
Romain et al	129 ± 21	711 ± 109	663 ± 136	1503
Buts et al	24 ± 3.5	220 ± 20	313 ± 16	557 ± 18

1.8.3 Polyamine metabolism in mammary gland

In mid to late pregnancy the mammary gland reaches its maximal concentration of epithelial gland cells, which is reflected by the increase in biosynthesis of polyamines during these two stages. The activity of the ODC enzyme has been shown to increase during pregnancy and early lactation in rat mammary gland (83). In addition, studies on polyamine metabolism in mammary gland suggest the metabolic conversion of polyamine to another by the increase in activity of other metabolic enzymes during pregnancy and lactation (83). Animal experiments have shown that mouse mammary gland possesses a transport and uptake system for putrescine, spermidine and spermine, and the uptake of these bioactive amines can be stimulated during lactation due to the effect of hormonal changes (84). It has also been shown that the cellular uptake of polyamine increases with the increase in polyamine concentration in the medium. The same study demonstrated that intravenous spermidine administration in mouse increased the level of spermidine in mammary gland within 48 hours of injection (81). The study points out the importance of exogenous sources of polyamines in determining the metabolic pathway of polyamines in mammary gland.

1.9 RATIONALE FOR THIS THESIS

Knowing the levels of polyamines (putrescine, spermidine and spermine) in different foods is of great interest due to the association of these bioactive compounds to health and disease. However, there is a lack of relevant information on their contents in foods, for example in the Swedish Food Database.

The significant biological roles of polyamines have been acknowledged in many reports. In addition, due to their physiological role in maintaining the function of body organs, dietary intake of polyamines has been shown to be necessary for all age groups, and not restricted to infants or growing children. Nevertheless, there are no recommendations for polyamine intake from foods. In epidemiological studies, a healthy diet has been identified, providing protection against several types of chronic disease (85, 86). It is of great importance to understand the link between healthy diet recommendations and polyamine intake.

Even though the occurrence of polyamines in human milk has been traditionally established (11), their concentrations in milk vary between studies (69, 70, 72, 82). Further, the contents of polyamines in human milk have also been reported to vary substantially during different stages of lactation (72). In addition, dietary intake has been hypothesized to have a significant influence on the polyamine concentration in breast milk (87). If dietary polyamines can have an influence on their contents in mother's milk, controlling diet during pregnancy and lactation would be of importance since polyamine intake from milk is essential for the newborn and infant.

The latest reports from the World Health Organization on breastfeeding in Sweden shows that the exclusive breastfeeding rate at 4 months is about 56% (88). This means that almost half of the children might be still fed on formulas. Comparing the levels of polyamines in formulas commonly used in Sweden with those in human milk is of importance.

2 AIMS OF THE THESIS

The general aim was to develop a polyamine database and use it to estimate polyamine intake among adolescents and lactating mothers, and to detect the levels of polyamines in human milk.

The specific aims were:

I. To develop a polyamine database by which polyamine intake and food contribution to this intake can be estimated, and to detect the levels of polyamines in Swedish dairy products.

II. To estimate polyamine intake and food contribution to this intake in comparison with the Swedish Nutrient Recommendations (SNO).

III. To determine polyamine concentrations in early human milk after preterm and full term deliveries, and in some formulas, and to study the association between mothers' dietary intake and the polyamine concentrations in breast milk.

IV. To investigate the effect of a weight reduction intervention program during pregnancy on the levels of polyamines in breast milk at different times of lactation.

"Quality means doing it right when no one is looking"
Henry Ford

3 METHODS

3.1 OVERALL STUDY DESIGN

Polyamine contents in foods were collected using an extensive literature search of databases (study I). Laboratory analyses using HPLC were performed to detect polyamine concentrations in dairy products (milk with different fat percentages, yogurt, cheeses and sour milk), different types of formulas, and in preterm and full term human milk from normal weight and obese mothers including a smaller group who had been subjected to a dietary intervention (studies I, III, and IV). The database developed on food polyamines was then used to estimate the intake of polyamines among adolescents in a cross-sectional study, in order to compare it with the intake from the ideal diet SNO (study II). It was also used to estimate polyamine intake among lactating mothers to associate their intake with the level of polyamines in milk (study III). Study IV was an intervention approach to investigate the effect of a dietary intervention program during pregnancy on the levels of polyamines in breast milk over a 2 month period of lactation. Figure 3 shows the design for the four studies, while table 2 shows the number of samples and the participants in the studies.

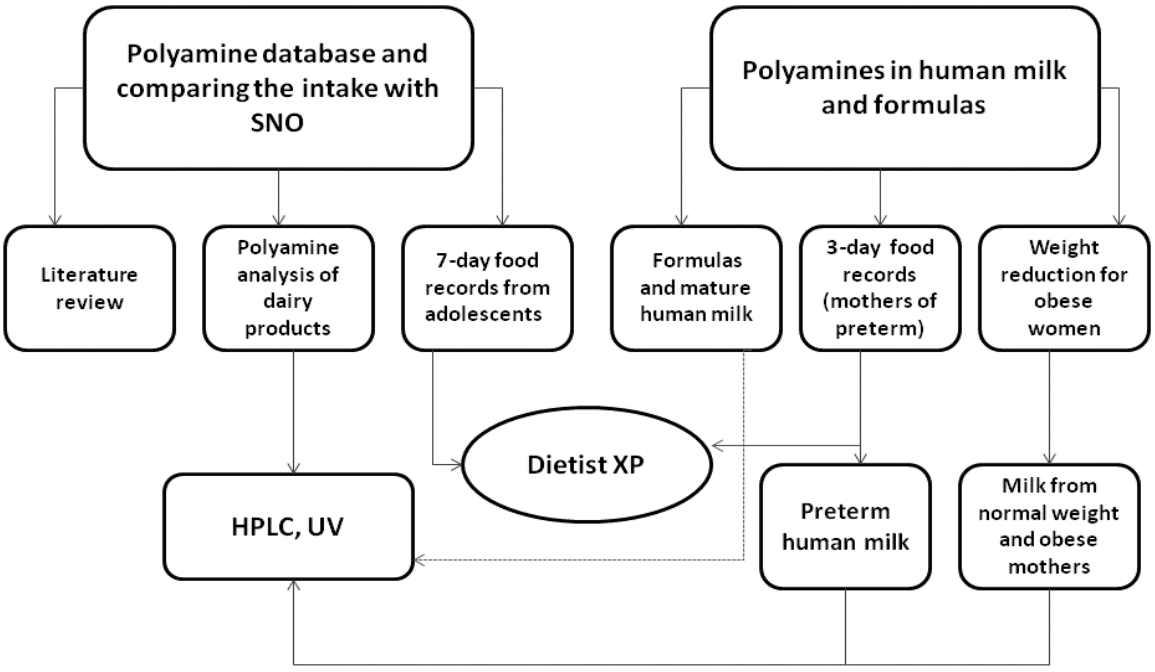


Figure 3. Study design

Table 2. Number of participants and samples used in the studies

	Study I	Study II	Study III	Study IV
Dairy products including milk, sour milk, cheeses and yogurt	5 samples of each	-	-	-
Adolescents volunteers who registered 7-day dietary records	-	93 adolescents	-	-
Preterm milk (6-10 days)	-	-	Milk samples obtained from 40 mothers	-
Full term milk at10 days	-	-	Milk samples obtained from 12 mothers	-
Full term milk at 3 days	-	-	-	43 human breast milk samples
Full term milk at 1 month	-	-	-	46 human breast milk samples
Full term milk at 2 months	-	-	-	41 human breast milk samples
Premature formula	-	-	3 samples of Enfalac	-
Infant formulas	-	-	3 samples of each type (Nan 1 and 2, and Semper 1 and 2)	-

3.2 DATABASE DEVELOPMENT AND DIETIST XP (STUDIES I AND II)

The database development begun with an extensive search on any reported data on polyamines in foods. The literature search was performed in PubMed, Web of Science, and SciFinder Scholar, and all papers published from 1986 to June 2009 were selected. Based on whether values on polyamines in the same food obtained from the same or several studies were different or extremely different, the mean or median were calculated. Polyamines in foods can be reported either in nanomoles or milligrams. All values were recalculated to mg/kg and the calculation was done based on each polyamine's molecular weight.

Dietist XP is a dietary program which is used for estimation of food, micro- and macronutrients, and energy intake. It is linked to the Swedish Food Database (Livsmedelsverket-National Food Administration, 2007, Uppsala) (50). Data on polyamines in each food were entered into the Dietist XP software version 3.0 (2007),

where putrescine, spermidine and spermine were assigned as additional food components besides the other nutrients. In Dietist XP, all values of polyamine contents are listed in mg/100 g of food.

The Swedish National Food Administration has estimated the portion size for 1334 foods. All portions in grams are available in the Swedish Food Database where the units in grams or deciliters can be found for most of the food available. In Dietist XP, the weight of the food per portion size, the weight in grams per deciliter for liquid food, and the amounts in grams for each spoon or cup are all obtained from the same data available in the Swedish Food Database (Livsmedelsverket, 2001). The concentration of each polyamine in foods per portion can then be estimated using the Dietist XP software. Portion size was considered due to the fact that portion size and frequency of consumption of food vary considerably, which means that a normal food frequency questionnaire would not necessarily detect the intake of products with high amounts of polyamines; for example sauerkraut or well fermented cheese.

According to the National Swedish Food Database classification of foods, all foods were categorized into food groups. Data on polyamine contents in foods were then aggregated to provide an average value for each polyamine in each food group. These are fruits, vegetables, dried pulses, cheese, bread and cereals, meat/fish/eggs, and potatoes. Dietist XP is designed to estimate the mean intake of each of these food groups.

3.3 STUDY SUBJECTS, POLYAMINE INTAKE ESTIMATION, AND DIETARY INTERVENTION (STUDIES II, III, AND IV)

The number of each study participants who either recorded intake or/and provided breast milk samples is shown in table 2.

3.3.1 Adolescent volunteers (study II)

A group of adolescent volunteers were randomly selected from schools in Eskilstuna, Strängnäs and Eksjö, Sweden, and asked on a voluntary basis to record their dietary intake using 7-day food records. Their mean age and BMI were 17.4 years and 21.6 ± 2.1 kg/m^2 for males, and 17.5 years and 21.1 ± 1.8 kg/m^2 for females. The adolescents

were provided with food scales and registration forms. In addition, instructions on how to record the intake were given by experienced nutritionists on the day before food intake started, and advice was available on the phone and in person throughout the week of registration. Dietary data were entered in Dietist XP for estimation of polyamine intake and the contribution of food groups to this intake. Fruits, berries, and fruit juice were categorized as fruit group. All vegetables including carrot and roots were categorized as group of vegetables. Potato was given a separate group, while the bread and cereal group covered bread, grains and corn products. The meat group covered all types of meat, fish and egg products. Cheese was counted as a separate group of the most commonly consumed types of cheese in Sweden.

3.3.2 Mothers to preterm infants (study III)

Forty Mothers who gave birth to preterm infants after 24-36 completed weeks of gestation were included. Their mean age was 29.8 ± 6 years, and BMI was 24.9 ± 3.9 kg/m^2. The mothers' gestational age was determined from the 1st day of the last menstrual period and verified by an early ultrasound examination. First parity was recorded in almost half of the mothers, while second and more were registered in 37 and 15%, respectively. These mothers provided both breast milk samples and 3-day dietary records, covering the sampling day. The mothers' dietary intakes were recorded during three consecutive days including one weekend day. All intakes were recorded after following detailed instructions from the dietician, but no dietary advice were given during pregnancy or lactation. Based on the mothers' dietary records, polyamine and amino acid intake were calculated using Dietist XP, version 3.0, 2007, Sweden.

3.3.3 Normal weight and obese women (studies III and IV)

Mothers with normal BMI (20.9 ± 0.9 kg/m^2) who gave birth to full term babies and provided breast milk samples at 10 days of lactation were selected for comparison with milk from mothers of preterm babies. Their mean age was 30.2 ± 3.1 years. For parity, 58% of the mothers had their first delivery, and 42% had their 2nd one.

Thirty obese pregnant women (BMI ≥ 30) were included at the first visit to the antenatal clinic in the first trimester, and ten of them participated in a dietary and physical activity intervention program aiming to control the weight. Dietary advice was

given according to the general Nordic recommendations (51) and given following the "plate model" which included a visual tool showing meals to promote healthy eating by using pictures and charts (89). To check whether the women followed the given instructions, they were asked to keep diary records of their food intake regularly.

In addition to the two obese groups, 20 mothers who had BMI $\leq$ 25 were also selected. The control groups did not participate in any intervention.

3.4 SAMPLES AND SAMPLE COLLECTION (STUDIES I, III, AND IV)

Polyamine analysis was performed on samples from dairy products (commercial milk, sour milk, yogurt, and cheese), human milk and formulas (table 2). In study I, all dairy products were purchased from markets at least one day before analysis. These products were representative for the Swedish mostly purchased dairy products, with the exception of Gamle Ole, the Danish cheese that was included in the analysis due to its long fermentation period. Each sample was kept in refrigerator at 4 ºC until next day. In study III, a representative group of infant formulas available in Sweden was chosen for polyamine analysis. These formulas were Enfalac Premature® (Mead Johnson Nutrition, Nijmegen, the Netherlands), Semper® 1 and 2 (Semper AB, Stockholm, Sweden; and Nan® 1 and 2 (Nestlé Nutrition, Vevey, Switzerland). For breast milk samples, at about 1 week after delivery, preterm milk was collected over 24 hours using an electric pump. The 24-hour milk was kept at 4ºC during collection, carefully blended and an aliquot of 5-10 ml was frozen at -70 until analysis. For full term breast milk, mothers were asked to collect breast milk after nursing the baby. These samples were obtained at different times of lactation; 3 days, 10 days, and at 1 and 2 months (studies III and IV). All breast milk samples were collected in a plastic vial and kept at -70 ºC until analysis.

3.5 POLYAMINE ANALYSIS (STUDIES I, III, AND IV)

For cow's milk, sour milk and yogurt, an aliquot of 5 ml was subjected to polyamine analysis, while in cheese 10 g was used from each type and homogenized as described previously (90). In human milk, polyamine analysis was performed on 1 ml of each sample. Each sample was spiked with a known amount of internal standard 1, 7-diaminoheptane. This was followed by polyamine extraction which was done by adding

a few milliliters of 0.6 N perchloric acid to the milk or dairy product depending on the initial amount of the sample before it was then kept at 4 °C for 1 hour. To separate the protein phase, the sample was centrifuged for 10 minutes at 4°C. Polyamine derivatization procedure was then performed by adding 5-10 µl Benzoyl chloride to the mixture, after keeping the pH > 13 by adding small amounts of 2 N NaOH. Polyamines were then extracted by washing the resulting solution twice with diethyl ether before it was kept under nitrogen for evaporation. The residue (benzoylated polyamines) was dissolved in 1 ml of 38% acetonitrile in water (the same solvent which was used as mobile phase). The resulting solution was then filtered using syringe filter, GF Millipore MA, USA and an aliquot of 50 µl of each sample was automatically injected onto the C_{18} column where the chromatographic separation took place. The chromatographic separation and polyamine detection were done by using High Performance Liquid Chromatography (HPLC, Waters 2690) equipped with a Nova-Pak C_{18} column (15×3.9 mm), and Waters UV detector 996. Data acquisition was accomplished with Millennium ^{32} Version 3.0 system. The entire HPLC run was under isocratic elution with a flow rate of 1 ml/min. All samples were subjected to replicate analysis.

Benzoylated polyamines were detected by UV absorption at 198 nm, as it has been shown to increase the absorbance by ca. 50 times when acetonitrile is used as solvent (91).

Polyamine identification was based on comparison between the retention times of polyamine standards. These were prepared at known concentrations from a stock solution and subjected to the same derivatization and extraction procedures.

Based on polyamine analysis in 10 runs of replicate samples, the inter-assay coefficient of variation (83) was found to be less than 10% for all polyamines.

3.6 CHEMICALS

Polyamine standards (putrescine, spermidine and spermine), Internal standard (1, 7 diaminoheptane), perchloric acid 70%, Benzoyl chloride 99%, and Acetonitrile 99.9% analytical grade were all purchased from Sigma-Aldrich (Chemie GmbH, Germany).

Diethyl ether was obtained from Scharlau Chemie S.A, Spain. NaOH was purchased from Merck KGaA, Germany.

3.7 STATISTICAL ANALYSIS

All statistical analyses were performed using Statistical Package for Social Science (SPSS versions 17.0 to 20.0, SPSS Inc, Chicago, IL, USA). Means and standard deviations or standard error of means were calculated for polyamines in dairy products (study I), polyamine intake per day (study II), and polyamine concentrations in formulas and human milk (studies III and IV). In study II, T-test was conducted to test the difference between male and female in terms of energy intake, food and polyamine intake. Testing the difference in the energy intake between day 1 and day 7 was performed using Paired-sample T-test. In study III, the mean differences in polyamine concentrations between groups of formulas and/or breast milk samples were computed using Mann-Whitney´s U-test. The association between dietary intake of polyamines and polyamine concentrations in breast milk was assessed as a correlation for continuous variables using Spearman correlation test. To control for polyamine intake, Partial correlation test was used for the association between amino acid intake and polyamines in milk. In study IV, ANOVA test and ANOVA repeated measures were performed to calculate the differences in polyamine concentrations in breast milk between different groups of mothers and between different days, respectively. The statistical significance level was set to 0.05.

3.8 ETHICS

Informed consent forms were obtained from adolescents and mothers to perform the studies (II, III, and IV). Approval to conduct the milk analysis was obtained from the Ethics Committee of the University of Gothenburg, Gothenburg, Sweden, and Ethics Committee of the Medical Faculty, Karolinska Institutet, Stockholm, Sweden.

"Facts are stubborn, but statistics are more pliable”
Mark Twain

4 RESULTS

4.1 POLYAMINES IN FOODS FROM THE DATABASE AND IN DAIRY PRODUCTS (STUDY I)

The concentration of each polyamine in foods was independent of the others. In the database developed, orange, orange juice and grapefruit juice, sauerkraut, cheddar matured cheeses, cod roe, soy sauce, and soy miso were the highest sources of putrescine. Spermidine was high in dry soy bean, chicken liver, green peas, corn, shell fish and blue cheese. High levels of spermine were found in most of the meat products (sausages, pork, chicken and turkey), some vegetables (pumpkin), and cheddar cheese. This polyamine, on the other hand, was found in low quantities in other types of foods, and frequently reported as not detected (see table 1 in paper1).

Polyamine distribution in different food groups is shown in figure 4. The group of cheese was the highest source of spermidine, while the fruit group was the highest in putrescine. Both food groups were the richest sources of total polyamine.

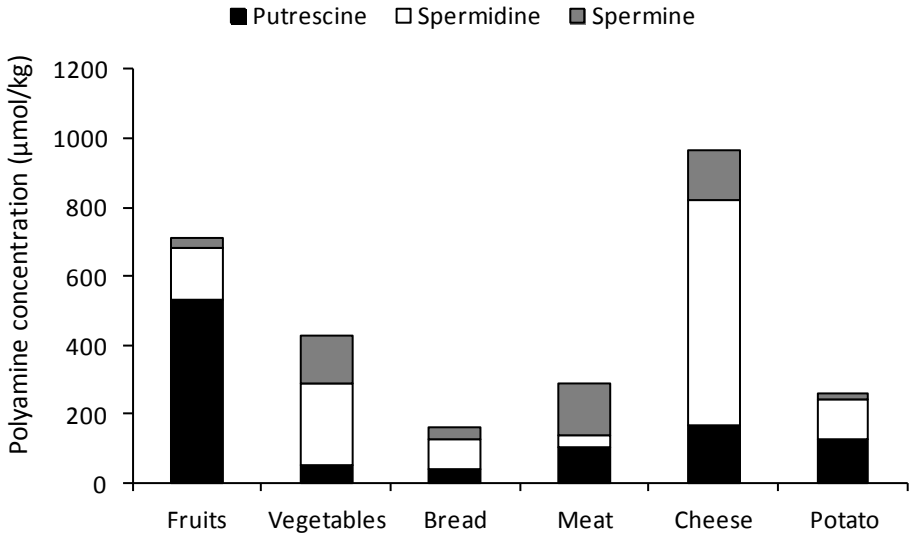


Figure 4. Polyamine concentrations in food groups according to the database

Figure 5 illustrates the results from our laboratory analysis of typical Swedish dairy products. The total polyamine level was higher in cheese with long maturation than other types (52.3 ± 5 mg/kg for putrescine, 1.2 ± 0.1 mg/kg for spermidine, and 2.6 ± 0.4 mg/kg for spermine). Unlike the milk, cheese that differs in fat percentage still had similar values of polyamines. The low fat milk and sour milk had the highest total polyamine contents, whereas the yogurt had the lowest contents. Sour milk had the highest mean putrescine contents. In general, cheese had higher total polyamine contents than the other products.

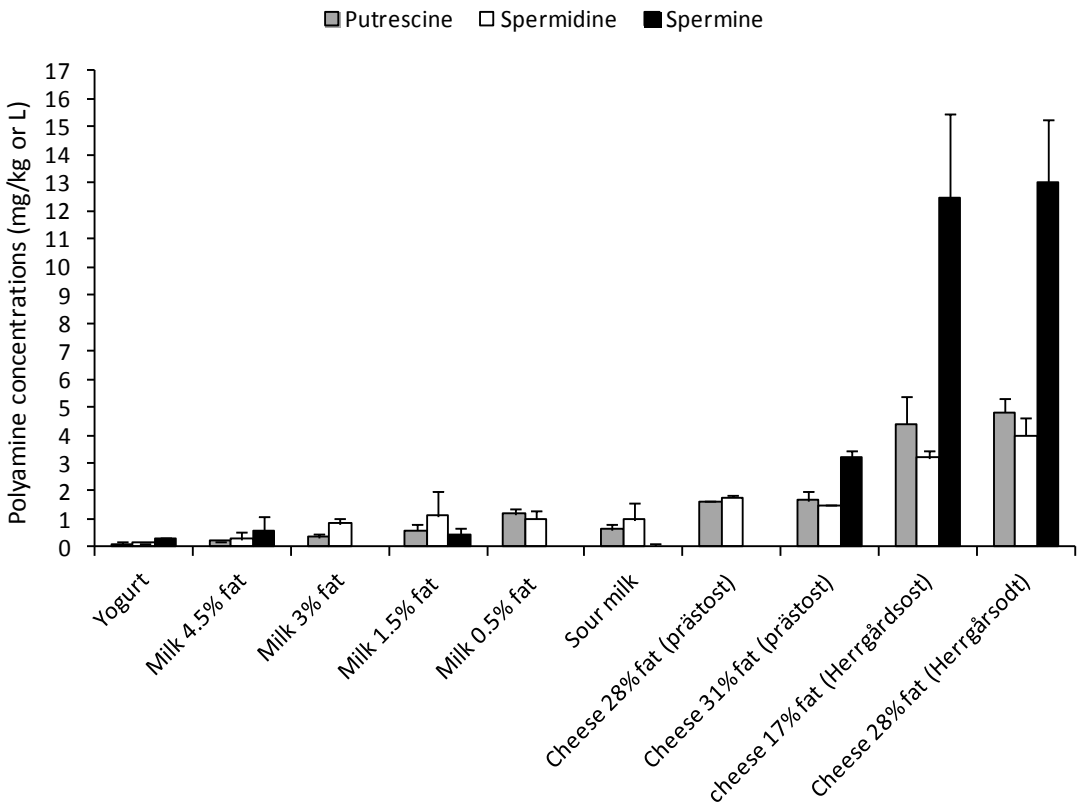


Figure 5. Polyamine concentrations in Swedish dairy products

4.2 FOOD AND POLYAMINE INTAKE AMONG ADOLESCENTS (STUDY II)

The average daily polyamine intake among adolescents was 316 µmol/day, representing 337µmol/day for males, and 303 µmol/day for females. There was a difference in spermidine and spermine intake between males and females ($p < 0.001$). This difference corresponded to a higher spermidine and spermine intake among males (75.7 and 43 µmol/day) than females (60.6 and 30.3 µmol/day).

According to the SNO, the list of foods constructed to meet the Swedish Nutrient Recommendations, the average total polyamine intake from this nutrient-wise ideal diet would be higher than the actual intake estimated for adolescents (figure 6). The total polyamine intake from SNO for males and females would be 530 µmol/day and 425 µmol/ day, respectively

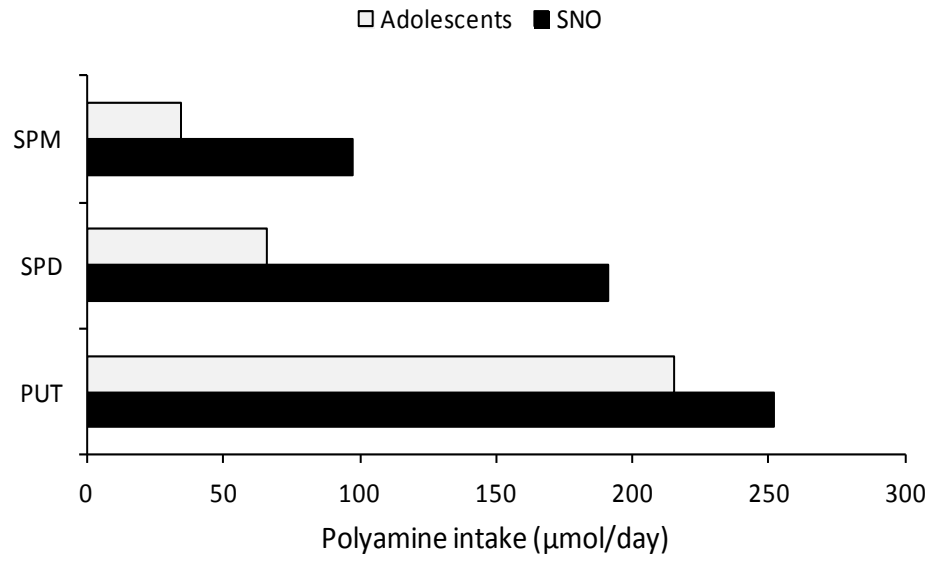


Figure 6. Polyamine intake of adolescents and from SNO

Figure 7 shows the intake of polyamines estimated from different food groups estimated for adolescents (A) and from the SNO (B).

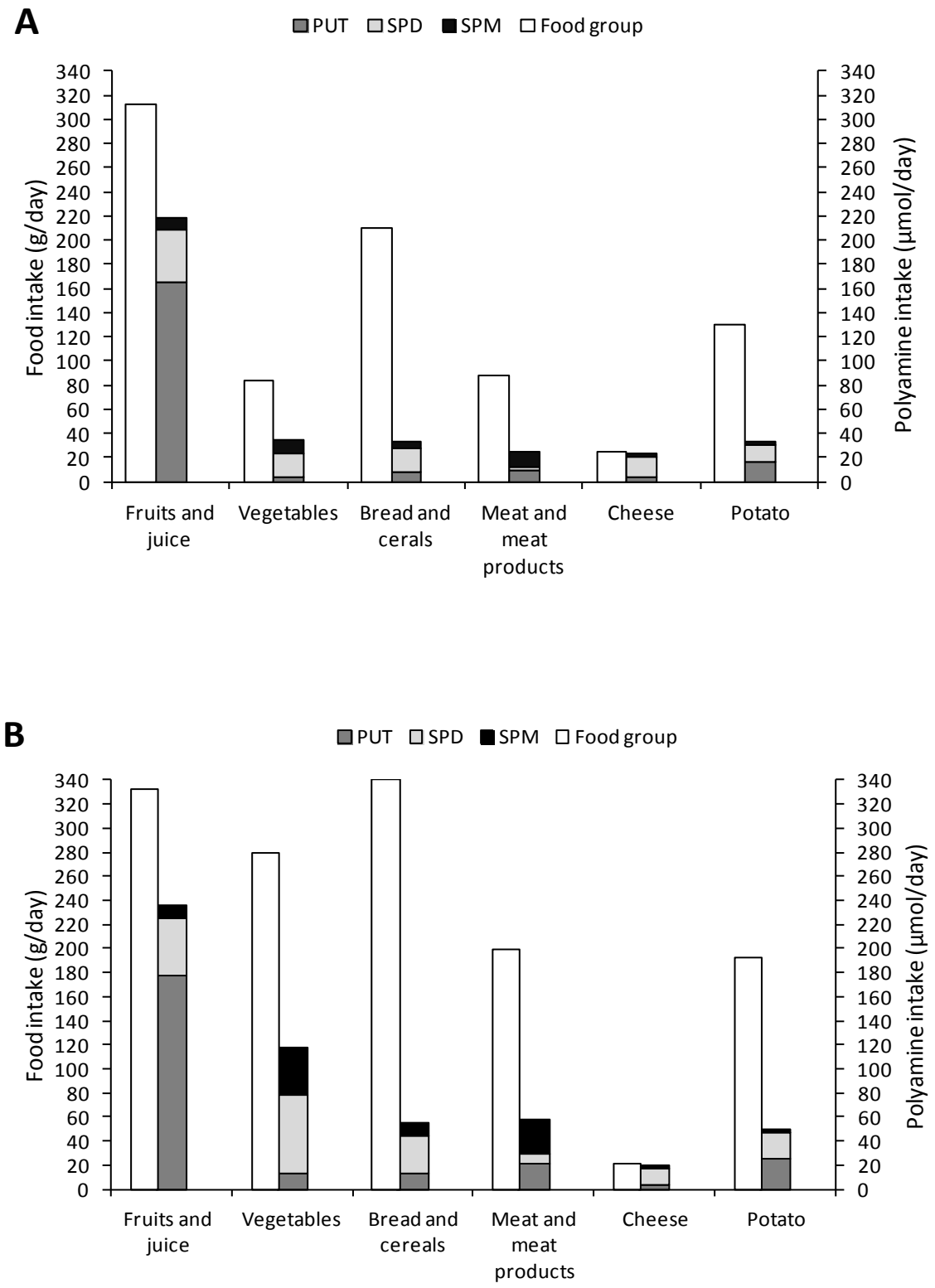


Figure 7. Food groups and polyamine intake estimated for adolescents (A) and from SNO (B)

4.3 POLYAMINES IN HUMAN MILK AND IN FORMULAS (STUDY III)

Polyamine concentrations in human milk and formulas are shown in figure 8. The concentrations of putrescine and spermidine were significantly higher in preterm than in full term milk ($p < 0.01$), while the spermine level was slightly lower in preterm than in full term milk. This would contribute to a higher mean (SD) total polyamine concentration in preterm (948.5 ± 400 nmol/dl) than the full term milk (713.3 ± 133 nmol/dl), ($p = 0.003$). In formulas, the highest putrescine and spermidine levels were observed in premature formula, contributing to a high total polyamine concentration in the latter compared with the other infant formulas ($p < 0.01$). The polyamine concentrations in human milk were significantly higher than those in corresponding formulas. Premature formula had significantly lower spermidine and spermine concentrations than preterm milk, while putrescine concentrations showed no significant difference.

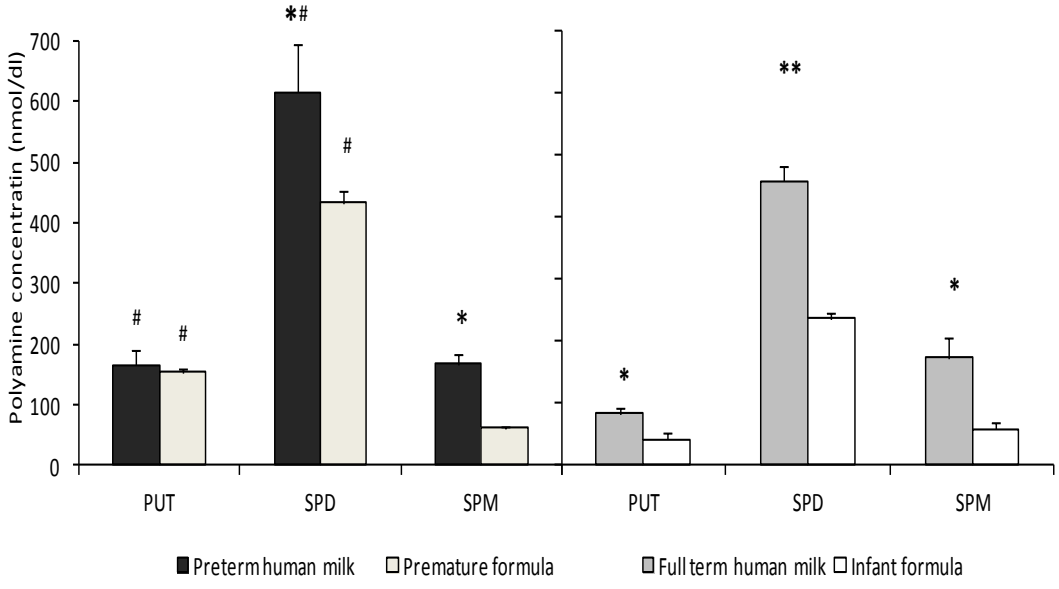


Figure 8. Polyamine concentrations in preterm (left panel) and full term (right panel) human milk compared to formulas. PUT = Putrescine, SPD = Spermidine, SPM = Spermine. * indicates difference between human milk and corresponding formula, * $p < 0.01$, ** $p < 0.001$. # ($p < 0.01$) indicates the difference between preterm and full term milk, and also between premature and infant formulas.

4.3.1 Polyamine intake among mothers

The mothers' intake is shown in table 3. More than 75% of daily putrescine intake was obtained from the consumption of oranges (30 ± 20 g) and orange juice (81 ± 60 g), contributing to 25 and 51% of putrescine intake, respectively. In addition, fruits and vegetables contributed to half of the spermidine intake. About 60% of the daily spermine intake originated from the mothers' consumption of bread and cereals, while only 35% of the spermine intake was from meat and meat products.

Table 3. Polyamine and amino acid intake according to the mothers' dietary records (n = 40)

Polyamine/amino acid	Median	Range
Amino acids		
Arginine (mg/d)	1379	340-2593
Methionine (mg/d)	779	115-1512
Polyamines		
Putrescine (µmol/d)	122.4	23.7-533.6
Spermidine (µmol/d)	82.6	27.8-167.2
Spermine (µmol/d)	41	17.3-85.3
Total polyamine (µmol/d)	246	83-729

4.3.2 Association between dietary intake and polyamine concentrations in breast milk

The intake of fruit and fruit juice was significantly associated with putrescine concentration in preterm milk ($r = 0.74$, $p < 0.001$).

Putrescine, spermidine, and spermine in breast milk were all significantly associated with the intake of each corresponding polyamine from food. In addition, there was an association between amino acid intake and spermine concentrations in breast milk, even when adjusted for spermine intake (table 4).

Table 4. The association between polyamine and amino acid intake and the concentrations in breast milk from mothers delivering preterm babies

Dietary intake	Putrescine in milk	Spermidine in milk	Spermine in milk
Putrescine	0.72**	0.45*	0.20
Spermidine	0.30	0.76**	0.40*
Spermine	0.07	0.40	0.53**
Arginine	0.20	0.04	0.60*
Methionine	0.20	0.04	0.50*

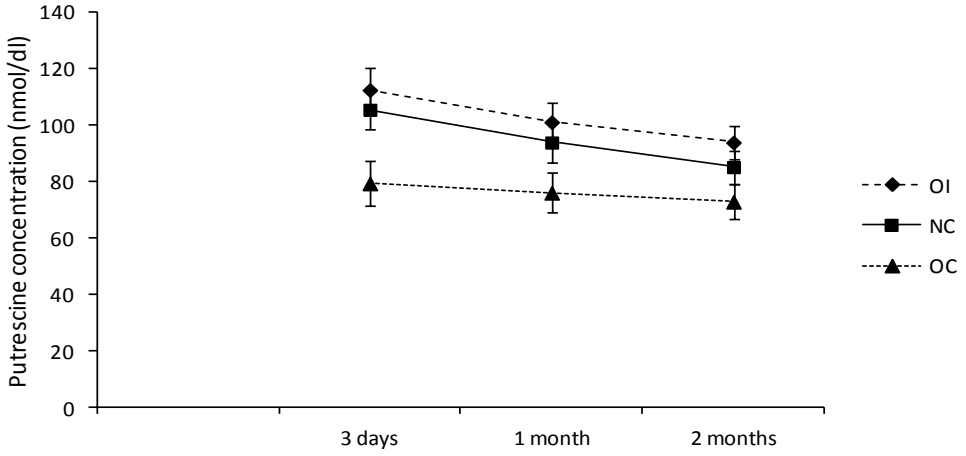
Each value represents correlation coefficient; *p≤0.01; **p<0.001

4.4 POLYAMINES IN BREAST MILK FROM OBESE MOTHERS (STUDY IV)

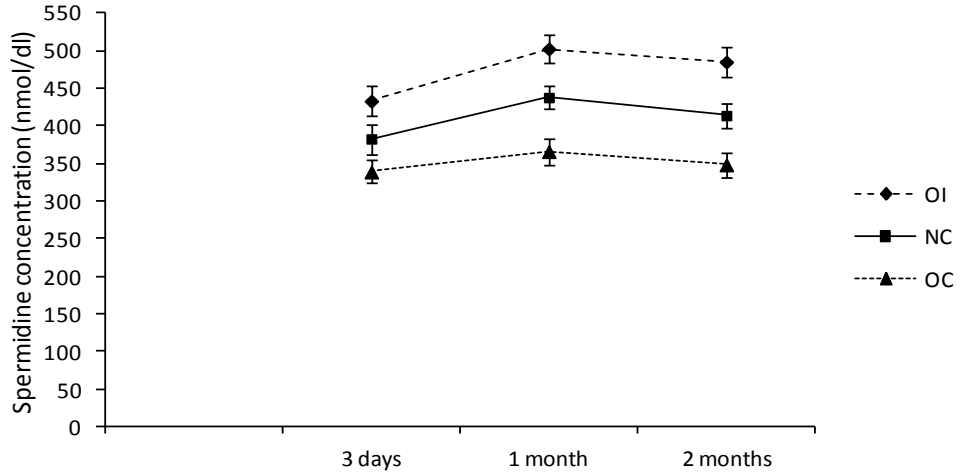
Putrescine and spermidine were significantly higher in the intervention group than the obese and normal weight control groups at all times ($p < 0.05$). This figure leads to a significantly higher mean total polyamine concentration (734 ± 29.8 nmol/dl) in breast milk from obese mothers after intervention than the obese control group (580.6 ± 23.8 nmol/dl). The lowest total polyamine concentration (567.6 ± 22.3 nmol/dl) was in milk from obese control mothers at 2 months of lactation ($p < 0.01$).

The longitudinal data shows that putrescine in breast milk decreased significantly over time (figure 9, A). Obese mothers subjected to dietary intervention had higher putrescine concentration in their milk at all times than the normal BMI and obese control groups (*P*-values were < 0.05 and < 0.01, respectively), while the latest group had the lowest milk putrescine concentrations. At 3 days lactation period, breast milk had the highest putrescine contents compared with other lactation periods for all groups ($p < 0.01$). Breast milk from the obese intervention group had also significantly higher spermidine contents (figure 9, B), while the obese control mothers had the lowest ($p < 0.01$). Spermidine concentrations were significantly higher at 1 month of lactation ($p < 0.01$). Figure 9, C shows that the highest spermine concentrations were at the first month of lactation in all groups ($p < 0.05$). At 2 months of lactation, the obese control mothers had the lowest spermine concentration. The difference in spermine concentrations between the 3 groups was not statistically significant.

A



B



C

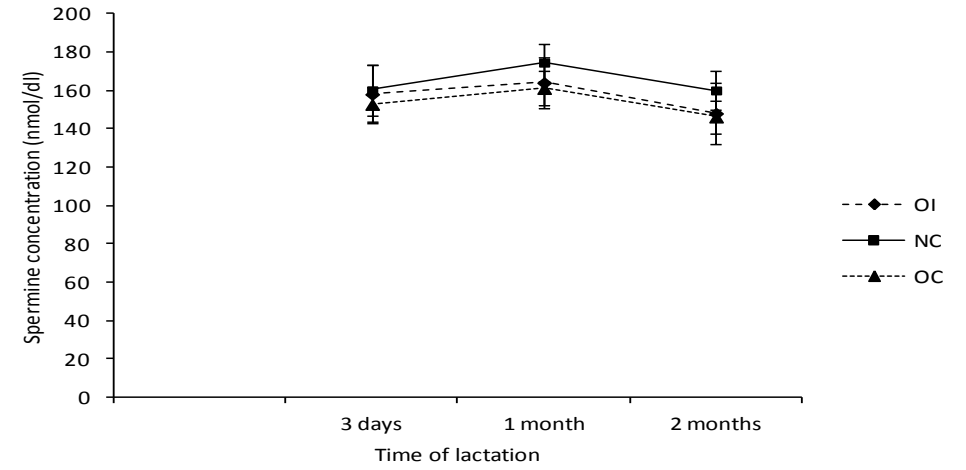


Figure 9. Polyamine concentrations in breast milk from control (OC) and intervention (OI) obese mothers, and normal weight control mothers at different times of lactation.

“There are no facts; only interpretations”
Nietzsche

5 DISCUSSION

5.1 POLYAMINE CONTENTS AND VARIATION IN FOODS (STUDY I)

The concentration of polyamines varies markedly in foods. This variation can be explained by several factors such as the storage condition of food, seasonal variations, food processing and cooking methods, and the difference in the analytical techniques (5, 47, 48, 71). In a study on the effect of cooking and storage of pig liver on the content of polyamines, spermidine and spermine concentrations have been shown to decrease to about 70% and 60-70% during cooking and storage, respectively (92). Putrescine concentration has been also shown to vary between studies (44, 93). In UK (44), putrescine in sausage has been reported to be more than 10 times higher than the one in sausage from Norway (93). In addition, the same polyamine has been shown to vary considerably from 2.6 mg/kg up to 416 mg/kg in 20 samples of Spanish fermented sausage (94). The same study has explained this variation by referring to the possible effect of the extent of the fermentation process as well as the metabolism of the fermentative bacteria used. In sauerkraut, which is rich in polyamines, the remarkable variation between samples (2.8-529 mg/kg) also can be explained by the difference in the bacterial flora during spontaneous fermentation (45). Similarly, the difference in the fermentation process and starter culture used for cheese production (93, 95, 96) might explain the difference in polyamine contents of cheese between studies (46, 90, 93, 97). In three different studies that have reported the content of polyamines in foods, putrescine concentration in tomato varied from only 1.2 to 122 mg/kg (44, 46, 97). Despite this variation in different foods, some studies have reported rather similar values for putrescine concentration in orange, spermidine in Japanese and Norwegian cheese, and spermine in raw lean beef (44, 46, 54, 93).

The Swedish dietary registration program Dietist XP, where polyamine contents are entered, is tailored to rank the food depending on content, either per portion or 100 milligrams of food. Using this function, cooked soybean and cow's liver were ranked as the highest sources of spermidine and spermine, respectively. Even though sauerkraut is rich in putrescine, in Dietist XP it came third after grape fruit and orange due to the difference in portion size. This could be useful for estimating how much polyamines a certain food would provide when the portion size is considered.

For example, grapefruit juice has lower contents of putrescine per gram than orange, but when ranked per portion size, it would appear to be a more important contributor to putrescine than orange, orange juice, and even sauerkraut.

When the highest polyamine sources in terms of portion size were compared with another study in the US that has listed the top 10 food sources (55), slight differences in the order of foods were found. These could be due to differences in the mean values for each polyamine that have been assigned for each type of food between the two databases, as well as to differences in portion size estimation.

A healthy diet consists of consuming moderate amounts of dairy products which are mostly cheese and yogurt (98). Epidemiological studies have shown that consumption of whole milk is positively associated with cardiovascular diseases (CVD), while, in some instances, cheese has been inversely associated with CVD (99, 100). Recent investigations have associated these observations to the higher content of polyamines in cheese, especially fermented one, than in milk (101). Moreover, the possible protective effect of polyamines against CVD has been explained by the potent anti-inflammatory effect of the natural polyamines (32, 33, 102). Cheese, which is one of the staple foods in a country like Sweden, has been reported to have higher polyamine concentrations than in cow's milk (90, 93, 103). The results from our laboratory analysis of polyamines in Swedish cheese and milk were concomitant with the previous reports. In addition, the values for polyamine concentrations in mature cheese were comparable to the ones that have been reported in Spanish hard cheese (90). During cheese ripening, casein is slowly degraded by proteolytic enzymes, which leads to an increase in the contents of free amino acids (104). By the action of bacterial decarboxylases, the free amino acids will be subjected to breakdown reactions and form the polyamine, which increase by extending the cheese ripening time (105). The high putrescine content in matured cheese is expected to be a result of the fermentation process. Herrgårdsost is a Swedish cheese that has higher putrescine than the other Swedish type named Prästost. This may be due to the short maturation period and the use of bacterial starter that are applied on Herrgårdsost.

5.2 POLYAMINE INTAKE (STUDIES II AND III)

There is increasing evidence that following a healthy diet is accompanied by high polyamine intake (56, 101). In an attempt to compare the actual intake of polyamines with the one estimated from SNO, it appeared that not only the intake of adolescents was lower than the "ideal" polyamine intake, but also the lactating mothers who delivered preterm babies had lower levels of intake than those from an ideal diet. The levels of polyamines in the diet of these mothers corresponded to the intake of Swedish adolescents, particularly the contribution of fruits and juice to more than 75% of putrescine intake and similarly the contribution of meat to 30-35% of spermine intake.

Vegetable consumption can contribute to a high polyamine intake due to the high content of spermidine and spermine (4, 106). The intake of vegetables among the adolescents was far below the recommendations from the SNO. On the other hand, fruit intake among adolescents was rather high and therefore contributed to almost half of the total polyamine intake. In UK, only 24% of polyamine intake was consumed from fruit (4), while in Japan the main contributor was the intake of vegetables, with about 26% of the total polyamine intake (54). Compared with the intakes from other studies, the adolescent's mean polyamine intake and median intake among mothers were both higher than the one estimated in Japan (200 µmol/day) (54). In the US, the total polyamine intake was 250 µmol/day, while in Europe the range of intake varied between 300 and 390 µmol/day (4, 106). Differences in dietary pattern can have a great impact on the amount of polyamine intake which varies between regions (3). A higher intake that exceeded 700 µmol of total polyamine/day was estimated in the Mediterranean regions (6). This would reflect the higher amount of polyamines estimated from a recommended Swedish diet when compared with the actual intake.

Out of the total intake of polyamines, putrescine constituted about 68%, while spermidine and spermine accounted for 21 and 11% of the total polyamine intake, respectively. Even though the intakes were different between studies, the percentage of contribution of each polyamine to the total intake were rather similar to those estimated in the American study where the contribution of putrescine, spermidine and spermine to the total polyamine intake have been reported to be 64, 22 and 14%, respectively (55).

The significantly higher intake of spermidine and spermine in males compared to females might be explained by the females' significantly lower intake of bread and meat products (*p*-values were 0.001 and 0.004, respectively).

It has been suggested that a high polyamine intake from foods might stimulate cancer cells (107). Several studies have focused on a polyamine-reduced diet and its role in cancer therapy (63, 64, 108). It has been reported that using a combination of a low-polyamine diet with polyamine antimetabolites and anticancer agents was an effective regimen in treatment of gastrointestinal cancer (107, 108). For instance, in patients with prostate cancer, polyamine restricted diet has been shown to be effective in maintaining performance status and pain control (61). Therefore, limiting polyamine intake has been suggested as a strategy for cancer treatment and prevention. However, epidemiologic studies have shown that a diet rich in vegetables and fruits is protective against chronic disease (85, 86) and that polyamines are considered essential during physiological growth. This also points at a generally healthy diet being rich in polyamines and that concerns regarding polyamine intake probably is relevant only in cancer treatment.

5.3 POLYAMINES IN HUMAN MILK AND FORMULAS (STUDIES III AND IV)

One of the several significant biological roles of polyamines in human milk is their involvement in protein synthesis (11, 17, 20). Our laboratory data showed that the concentration of polyamines in breast milk of mothers who delivered preterm babies is significantly higher than in breast milk from those delivering full-term. In addition, it is well documented that preterm human milk has significantly higher protein levels than in full term human milk (109, 110). Sanguansermsri et al. (11) has shown that protein contents of milk seemed to run parallel with the level of polyamines, particularly putrescine.

We also found significantly higher putrescine concentrations in preterm milk (165.6 nmol/dl) when compared to full term milk (82.4 nmol/dl). Buts et al. (72) and Pollack et al. (69) have reported lower values for putrescine concentration (24 and 33.8 nmol/dl, respectively). Even though putrescine levels reported by Darhout et al. (82)

and Romain et al. (70) (77 and 129 nmol/dl, respectively) appeared to be rather higher than the previously reported concentrations, they are still lower than the concentrations we found in preterm human milk. Therefore, it is highly possible that the presence of higher amounts of polyamines in preterm milk could be associated with the need for these bioactive compounds for more protein synthesis at this early neonatal stage. This variation in putrescine concentration may be also due to genetic and/or dietary variability (70).

We were able to show that the content of polyamines in preterm human milk was associated positively with the mother's dietary intake. Our results confirm the previously revealed effect of dietary polyamines and amino acids on polyamine concentrations in rat's milk (81). Peulen showed that feeding rats with spermidine, spermine and amino acids induced an increase in the level of polyamines in rat milk, suggesting that polyamines in milk are at least partly dependent on dietary intake.

In mice mammary gland, the recovery and the uptake of exogenous polyamines have been studied at cellular level (84). The study showed that within 24-48 hours, more than 90% of spermidine was recovered in their initial form, while two thirds of the putrescine was converted to spermidine. Such findings support the association between putrescine and the intake and also with the spermidine level in breast milk, which we found in our study.

The effect of dietary intake on the contents of polyamine in milk did not seem to be only related to the intake of polyamines. We were also able to find significant correlations between the intakes of arginine and methionine and the concentration of spermine in breast milk. Arginine, which is a semi-essential amino acid, can be found in animal food sources like cheese, beef, poultry, and seafood. Chicken and fish contain relatively higher amounts of methionine compared to other foods. In addition, these foods are considered to be good sources of spermine. Therefore, controlling for the mothers' intake of spermine from these foods was considered when we studied the correlation between intake of each amino acid and spermine content in breast milk.

Oral administration of spermidine to neonatal rats has been shown to accelerate small intestinal maturation and thereby reducing the risk of food allergy (73, 75). In addition, epidemiological data has shown that the variation between mothers in breast milk

content of polyamines could lead to unfavourable health consequences in children, even later in life. For instance, mothers to children who have been diagnosed to be allergic to certain foods had produced breast milk with significantly lower contents of spermidine and spermine during lactation when compared with mothers to non-allergic children (78). Thus, our findings suggest that mother's diet is important in modifying the content of polyamines in breast milk and thereby determining the health and physiological condition of the child.

In addition to diet, the nutritional status of the mother can have an effect on the composition of milk and could lead to variation in polyamine contents (87). This is probably due to the wide range of polyamines in different foods (3). Several studies have suggested a role for dietary polyamines in prevention of chronic diseases that are associated with obesity (6, 15, 56). In addition, the high contents of polyamine in some fruits and vegetables, which are associated negatively with obesity, could be an assumption that obese women eat less polyamines than non-obese ones.

While the effect of a diet rich in polyamines has recently been examined on blood polyamine levels in both human and animals (35), to our knowledge, our study was the first to examine the effect of weight reduction intervention program on polyamines in human breast milk. A recent study on the Mediterranean diet and polyamine intake has reported that the intakes of fruit, vegetables, seafood, and cheese were all strongly and positively associated with the amount of polyamines consumed (56). In contrast, processed food and high sugar and fat products contain low amounts of polyamine, and have been shown to have no or negative association with polyamine intake (56). The higher polyamine concentration in breast milk from obese women with intervention than the control group may reflect an increased intake of fruits and vegetables and a reduction of junk food, which – together with regular meals - was the main dietary objective of this intervention.

In addition to the effect of dietary intake, the significantly lower contents of putrescine and spermidine in breast milk from the control group of obese mothers could be explained by other biological factors. For instance, while the uptake and accumulation of polyamines in mammary glands has been shown to be enhanced by prolactin (84) the secretion of this hormone has been shown to be lower in obese subjects during pregnancy, both in human and animals (111).

The content of polyamine changes over the lactation period. These changes have been seen as a reflection to the enhanced metabolic activities and protein synthesis rate in the milk of the mammal species (72). The pattern of the decline in putrescine concentrations during lactation was corroborating earlier studies. These have shown lower putrescine contents in breast milk by the end of the second month of lactation (11, 70). Moreover, even lower putrescine levels have been found in breast milk from mothers with low socio-economic conditions (11). A recent ecological study on polyamine intake and gross domestic product has reported that socioeconomic status is associated not only with dietary pattern in general but also with the intake of polyamines in particular, which was higher among the high socioeconomic group (3).

Even though there are no recommendations to either polyamine intake during infancy or the amounts in human breast milk, animal studies have shown that the bioactive effect of polyamine is dose dependent (112), and that a total polyamine intake of 3500 nmol/day would theoretically exert biological effect and cellular function (43, 112). Assuming that neonates and infants consume between 500 to 700 ml of human breast milk per day, all breast milk from obese mothers with intervention as well as normal weight control group would provide a total daily polyamine intake that exceeds the biologically active suggested concentration. In contrast, only 25% of the control group of obese mothers had breast milk providing theoretically lower polyamine intake per day than the suggested biologically active concentration.

Despite the variation between mothers, human milk has been shown to contain sufficient bioactive amines to sustain biological function and promote cellular growth in comparison with bovine milk and infant formula (75). We were also able to show that polyamine concentrations were higher in human milk than in the corresponding formulas, and higher in preterm than full term milk. In addition, spermidine concentration in infant formula exceeded the levels of putrescine and spermine. The same pattern was seen in another study on polyamines in infant formulas (69). Spermidine in preterm human milk showed a similar pattern but had even higher spermidine concentrations than in premature and infant formulas.

Oral administration of spermidine to neonatal rats has been shown to accelerate small intestinal maturation and thereby reducing the risk of food allergy (73, 75). Romain et al. (70) has suggested that adding polyamines to infant formula might reduce the

likelihood of developing allergy in children. Even though the addition of polyamines to infant formulas up to levels that can be similar to human milk has been estimated to be non-toxic (9, 113), it is still questionable whether such improvement would compensate for human milk and sustain biological function. Further, the lack of bifidus bacteria in the colon of formula-fed babies (114), who may show less bacterial biodegradative synthesis of putrescine from ornithine and arginine (115), may not fulfil the requirements of polyamines to maintain growth and maturation of specific organs (82).

Animal studies have shown that orally administrated polyamines have stimulated gut growth and carbohydrate absorption neonatal pigs and calves (116, 117). Dietary putrescine has been shown to enhance whole-body growth in chicks (118). In rats, exogenous spermine and spermidine both have been shown to stimulate the activity of disaccharidase enzyme (119, 120). In addition, polyamines have been shown to promote gut hypertrophy and inhibit gastric acid secretion (37), and promote ulcer healing (38, 69). These different roles would imply the importance of each particular polyamine in human milk and foods during not only early growth and development, but also for adults and elderly.

5.4 METHODOLOGICAL CONSIDERATIONS

There was only a limited number of dairy products and the formulas obtained for polyamine analysis, however, the products chosen were the most commonly purchased brands in Sweden, and the variation in the results from our analysis was rather small.

Polyamine intake was estimated only among adolescents, and therefore cannot be seen as representative for the Swedish population. Furthermore, due to the low number of participants, we cannot say that the results are representative for all Swedish adolescents. However, the 7-day dietary records were conducted with support by a trained nutritionist and according to the energy intake, under-registration occurred only in a few participants. Therefore, we can say that the results were representative for the groups that were investigated.

The aggregation that was made for each food group to give an average estimated value for each polyamine has some limitations due to the fact that within the food groups there are wide variations in the polyamine contents. For instance, in the group of fruits,

orange was reported to have about 20 times higher putrescine than that in apple (10). Therefore, having one polyamine value for one food group was just an estimation and does not give any exact value for the average polyamine concentration in that specific food. Nevertheless, the values of polyamine intake calculated from foods consumed compared to the values of polyamine intake that were estimated from the food groups showed some similarity, especially for the total polyamine intake among males.

The estimated polyamine intake was based on data on polyamine contents of foods that was collected from different studies. These studies showed some variations as explained previously. In addition, there is still lack of information on contents of polyamines in some food groups. Therefore, the adolescents and mothers' polyamine intake is only an estimate to their actual intake which might be either over- or underestimated in this study.

The large range of polyamines in human milk could be explained by the variability between the mothers which can be due to many influencing factors. Here in this study, we were only able to confirm that dietary polyamine intake was associated with the content in the breast milk.

The number of obese women with intervention was rather small; however, milk samples were obtained from at least three lactation periods from each mother. This will allow for better comparison when looking at the difference in polyamine levels in milk using longitudinal data.

Human and cow's milk contain polyamine oxidase (PAO) that can induce a decrease in polyamine concentration over time (121). We took special care of the milk samples either by performing polyamine analysis after short period of obtaining the milk from the market, or by maintaining human milk samples and formulas in at least -70 ºC until analysis.

6 CONCLUSIONS

The database provides information for other researchers in their quest for information on polyamines in foods and also allows for estimation of polyamine intake. Polyamine concentrations in Swedish dairy products were detected to allow for adaptation to the Swedish population when polyamine intake is estimated.

This variation in reported polyamine levels between different studies and even within the same food, underlines the needs for caution when interpreting the results from literature. Therefore, further analysis of polyamines in different Swedish foods may be required in order to tailor the database to be used on population level. This can also be used in clinical studies where polyamine intake can be estimated in relation to both health and disease.

Among the adolescents, the estimated total dietary polyamine intake, for which fruits were the major contributor, was similar to previous reports from Europe. According to the Swedish Nutrition Recommendations Objectified, the total polyamine intake appears to be higher in an ideal diet than the current estimated intake among adolescents. In addition, by following the SNO, the contribution to the total and individual polyamine intake seemed to be stemming mainly from foods of plant origin.

The higher concentrations of polyamine in preterm than full term human milk might reflect the higher needs of preterm babies, as also reflected in the well known higher protein content of preterm milk. The impact of lower polyamine concentrations in formulas than in human milk for the development of allergy or other intestinal disorders needs further studies. Furthermore, the role of the intestinal flora in polyamine synthesis has been previously acknowledged (122). To what extent the levels of faecal polyamines as well as the amounts absorbed and those synthesised in the intestinal lumen can be affected by external polyamines consumed from human milk needs further studies.

The strong association between the content of polyamines in breast milk and mother´s dietary intake points at the importance of a healthy diet and a good nutritional status before pregnancy as well as of dietary advice during lactation, especially when we

showed that a long term dietary intervention seemed to be effective in increasing polyamine concentrations in obese women´s breast milk. Further studies are needed to investigate if these differences are of importance for a healthy development of the infants, and whether the low contents of polyamines in breast milk of obese mothers are associated with other hormonal and physiological factors, in addition to food choice.

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