STUDIES ON THE AETIOPATHOGENESIS OF TYPE 1 AUTOIMMUNE DIABETES

By

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Doctor of Philosophy

University of Western Sydney

2006
“For by doubting we are led to question and by questioning we arrive at the truth” Peter Abelard (1099-1142)
Statement of Authentication

The work presented in this thesis is, to the best of the researcher's knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material either in whole or in part, for a degree at this or any other institution.

Hayder A. Al-Domi ...........................................
Ethical Approval

The Human Research Ethics Committee of the University of Western Sydney as well as the authorities of all Jordanian participating centres (The National Centre for Diabetes, Endocrinology and Genetics, Princess Rahmah Children’s Hospital, and Al-Zahrawi Medical Laboratory) approved all studies described in this thesis. All subjects, or their parents when appropriate, gave written informed consent, or verbal witnessed consent when appropriate, for the studies undertaken.
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<td>a peptide fragment of 13 amino acids of bovine serum albumin</td>
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<td>ADA</td>
<td>American Diabetes Association</td>
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<td>ALA</td>
<td>alpha-lactalbumin</td>
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<tr>
<td>AU</td>
<td>absorbance unit</td>
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<tr>
<td>BB-rat</td>
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<td>BI</td>
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<td>DERI</td>
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<td>DIAMOND</td>
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<td>DC</td>
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<td>DIPP</td>
<td>Type 1 Diabetes Prediction and Prevention Study</td>
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<td>DPT-1</td>
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<td>GAD</td>
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<td>ELISA</td>
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<td>infant starter formula</td>
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<td>milk protein solutions</td>
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<td>non-diabetic children</td>
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<td>non-obese diabetic mouse</td>
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<td>OD</td>
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<td>PHFBM</td>
<td>pasteurised homogenised fresh bovine milk</td>
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List of publications and conference presentations

The following publications (under review) have been derived from this thesis:


Conference presentations

• Al-Domi H., Jones, R., and Bergan, J. Possible diabetogenicity of modified and denatured bovine milk proteins. In: Second annual College of Science, Technology and Environment Innovation Conference. Sydney, Australia: University of Western Sydney; 7-8 June 2005.


• Invited lecture: Type 1 autoimmune diabetes: Controversy and future perspectives. Agriculture Faculty summer seminars programme, Department of Nutrition and Food Technology, Jordan University of Science and Technology, Jordan, November 21, 2005.
Abstract

Type 1 autoimmune diabetes mellitus (T1ADM) is a serious worldwide health problem. A minimum of three million individuals worldwide have the disease for the majority onset occurs during childhood. The disease is widely considered as a complex, chronic, metabolic, slowly progressive, polygenic, inflammatory organ-specific autoimmune-mediated disorder induced by activated autoreactive T cells. The natural history of the disease involves four phases of variable duration and is characterised by genetically determined susceptibility, occurrence of autoimmune markers, and selective aggressive destruction of insulin-producing pancreatic beta cells.

There is a strong assertion that every case of every disease has both genetic and environmental causes. Nevertheless, the exact aetiopathogenesis of autoimmune diabetes is not yet fully understood, what is evident however, is that autoimmune diabetes is a multifactorial disorder featured by a complex interplay between genetic and non-genetic (dietary and non-dietary) environmental factors which trigger the destruction of insulin producing pancreatic beta cells. Studies on the association between early exposure to foreign environmental triggering factors and the development of the disease remain discrepant and widely controversial.

Worldwide geographic variation in the disease occurrence among young children was significantly associated with global variations in mean and relative changes in both total milk imports and the amount of milk proteins consumed. This was influenced by the socioeconomic status of populations. Evidence on the diversity of milk protein solutions as well as the effect of modification and thermal processing on these proteins was established by the identification of changes in their spectral profiles and chromatographic profiles.
Studies on type 1 autoimmune diabetes in Jordanian children with diabetes are very limited. Baseline dietary profile, breastfeeding and early infant feeding practices, susceptibility relationship with ABO and Rh (D) blood system, and secretory and humoral immune responses to native, modified and thermally processed bovine serum albumin with bovine insulin were investigated in 50 young Jordanian diabetic children aged 14 years or less and 50 age and sex matched controls.

Breastfeeding in Jordan is common and is usually extended for a minimum of one year for the majority of children. In this study, diabetic and non-diabetic children were breastfed for a period of time with a higher proportion of diabetic children received no bottle-feeding in their infancy. Disease diagnosis age was approximately at six years of age, and was mainly in winter with female predominance.

Dietary compliance in diabetic children was very poor with signs of undernutrition in both groups. Better nutritional counselling and standardisation of national dietary guidelines as well as establishing a national diabetes registry may improve their nutritional status. Blood group O+ was predominant in diabetic children and a higher proportion of NDC had Rh (D) negative blood groups with gender variation.

Although bovine milk proteins are among the most investigated possible diabetogens, few studies have examined the modification and thermal effect on its immunogenicity. Changes in the mucosal architecture as well as interactions between different dietary (native, modified, and thermally processed), and non-dietary (normal flora and viral) factors coexisting in the gut environment may either boost or suppress the autoimmune process. Concomitant investigation of these factors may constitute a new ground for identifying possible primary environmental factors involved in triggering the pancreatic autoimmune process.
The newly developed saliva-based ELISA is non-invasive, rapid, and convenient method and constitutes a useful proxy of immune response studies in young diabetic children. The heat processing of bovine proteins at various temperatures as well as exposure times has caused substantial change in the secretory and humoral immune responses with significant variations. Immune responses were neither limited to diabetic patients nor associated with the breastfeeding or early infant feeding practices with the higher proportion of diabetic children receiving no bottle-feeding in their early infancy. This may reflect an unspecific defect of the immune system in young Jordanian children. Considerable variations between low and high-risk populations require further investigation.

Enhancing our understanding of the biological history of the preclinical stage of the disease, achieving ample insights into disease immunopathogenesis, and identification of persons for prevention trials could lead to more efficient early diagnosis prior to the manifestation of the disease. Identifying the nature and timing of environmental factors as well as genetic factors that may predispose to the initiation of the disease should continue. Eventually, the benefits to individuals and the world society will be enormous and that should provide the momentum to continue clinical studies on therapeutic intervention.
Type 1 diabetes mellitus remains a serious, costly, and rising worldwide health problem resulting in substantial morbidity and mortality. Although the majority of people with type 1 diabetes have autoimmune aetiology, the aetiopathogenesis of autoimmune diabetes as a form of diabetes for immune-mediated diabetes remains unknown. Understanding the aetiopathogenesis of autoimmune diabetes requires setting down the nature of the triggering factors, target autoantigens and the effector mechanisms. The natural history of the disease involves four phases of variable duration. In addition to genetic susceptibility linkage to or protection from the disease, environmental factors are widely implicated in initiating the pancreatic autoimmune process. Nonetheless, given the complexity of the aetiology of autoimmune diabetes and that multiple islet molecules are the target of autoimmune process, it is apparent that a number of various mechanisms of beta cell destruction are operative in type 1 autoimmune diabetes.

Short-term breastfeeding and early introduction of bovine milk-based infant formula may predispose young genetically susceptible children to progressive signs of beta cell destruction. Although dietary proteins, particularly bovine milk proteins are among the most investigated putative non-genetic determinants implicated in the disease aetiopathogenesis, the timing, nature, and characteristics of environmental diabetogenic factors remain unknown and no causal links have been established unequivocally. Therefore, it is important to embark on identifying changes that persevere up to the initiation of the destruction of insulin secreting pancreatic beta cells, not only to minimise or stop the progression of the ongoing process, but also to prevent the destruction process.
Rationale of the study

It is well recognised that every disease has both genetic and environmental causes. There are indications that environmental factors may play a crucial role over a limited period of time in genetically susceptible individuals to initiate the pancreatic insulin producing beta-cells autoimmune process. The nature of triggering factors, target autoantigens, and the effector mechanisms are not clearly understood. No causal links between environmental triggering factors and the pancreatic autoimmune process have yet been identified unequivocally.

Although the occurrence of autoimmune diabetes in Jordan is increasing by approximately one percent annually, studies on diabetes in young Jordanian children remain limited. Therefore, the rationale of this study hinges upon establishing a base-line dietary profile, and determining possible humoral and secretory immune response to native and heat processed bovine serum albumin modified with bovine insulin in young Jordanian children with diabetes.

To minimise the increasing occurrence of the disease through proposing safe and inexpensive preventative interventions of the disease, this study is directed at improving the understanding of the aetiopathogenesis of the disease by participating in identifying potential primary environmental triggering factors. Obviously, the health care system is a vital element in every society to maintain people’s health of the highest standard.
Objectives of the study

The objectives of the present study were:

- to study possible associations between both worldwide mean and relative change in both imported milk excluding butter and milk protein consumption, and both mean and global increase in the disease occurrence;

- to examine the diversity of native bovine milk proteins and the effect of modification and heat processing on those proteins by studying changes in their spectral and chromatographic profiles;

- to establish a baseline dietary profile of young diabetic and non-diabetic Jordanian children aged 14 years or less in terms of breastfeeding and early infant feeding practices;

- to determine serum IgG antibody titre levels produced to native bovine serum albumin as well as to native and heat processed modified bovine serum albumin with bovine insulin in diabetic Jordanians aged 14 years or less;

- to develop an a new non-invasive enzyme linked immunosorbent assay (ELISA) for determination of saliva IgA antibody titre levels produced to both native bovine serum albumin, and native and heat processed modified bovine serum albumin with bovine insulin compared to serum IgG antibody titre levels in diabetic Jordanians aged 14 years or less; and
• to establish a ‘susceptibility relationship’ between ABO and Rh blood systems and the development of type 1 autoimmune diabetes mellitus in young diabetic Jordanian children.
The initiation and progression to type 1 autoimmune diabetes in genetically susceptible individuals involve a number of mechanisms of beta cell destruction. The interruption of multiple mechanisms, rather than one single pathway, leading to the destruction of beta cell can be the key method to prevent type 1 autoimmune diabetes. The scope of the present study is exemplified in the following two Mind Maps. The first Mind Map shows the main processes implicated in the natural history of type 1 autoimmune diabetes, and the second Mind Map illustrates the design of the protein studies.
Mind map I: The natural history of type 1 autoimmune diabetes

(Modified from Rewers et al 2005)
Mind map II: Protein studies
Overview of the thesis

This thesis contains seven chapters compiled in a manuscript format. Chapter 1 provides a historical scientific background and a framework needed to understand the aetiopathogenesis of the disease. Chapter 2 studies possible association between worldwide mean and relative change in both imported milk and milk protein consumption, and mean and global increase in the disease occurrence. Chapter 3 embarks on establishing baseline data on the dietary profile of Jordanian children with diabetes aged 14 years or younger in terms of breastfeeding and early infant feeding practices. Chapter 4 investigates susceptibility association for the proportion of ABO and Rh (D) blood systems in young Jordanians with diabetes. Chapter 5 aims at examining the heterogeneity of native, modified and heat processed bovine milk proteins by studying changes in their spectral and chromatographic profiles compared to that of human breast milk. Chapter 6 focuses on developing a new non-invasive saliva-based enzyme linked immunosorbent assay to determine the secretory immune response as well as humoral immune response to native, modified and heat processed bovine milk diabetogens in young Jordanian diabetic children with regard to their early feeding practices. Chapter 7 suggests the main aspects of future studies. Chapter 8 concludes the work.
1 Review of the literature
Diabetogenicity of bovine milk proteins: A puzzling link

1.1 Introduction

Autoimmune diseases are serious and costly disorders. They include an organ and a non-organ-specific diseases (Roitt et al. 2001; Ghaffar 2004). In addition to thyroiditis, Graves disease, Addison disease and polyglandular syndromes, type 1 diabetes mellitus is an autoimmune endocrine disease (Baker 1997). It is a worldwide life-threatening serious and complex lifelong health condition, which results in substantial morbidity and mortality despite great advances in disease control and treatment interventions (Rewers and Klingensmith 1997). It ranks third among the most widespread of severe chronic childhood diseases.

Nearly half of the world’s 200,000 individuals annually diagnosed with type 1 diabetes are children (International Diabetes Federation 2003). The disease occurrence increases with age in different paediatric age groups (Karvonen et al. 2000), affecting 0.5-1% of the general population throughout its lifetime, and is marked by obvious variation (Rewers and Klingensmith 1997; Onkamo et al. 1999). Geographic variation in risk genotypes for T1ADM (Kukko et al. 2004) and 350-fold worldwide variation in the disease occurrence rate among and within major ethnic and racial groups appear to follow a global ethnic and racial distribution (Karvonen et al. 2000). This indicates an important interplay between genetic susceptibility and environmental factors in the initiation and development of the disease.
Type 1 diabetes, an immune-mediated complex chronic metabolic disorder of multiple unknown aetiology is more dangerous than other forms of diabetes and is a direct life threat to persons with the disease (Bogardus and Lillioja 1992; Bach 1994; Williams 1999). Even with good care, diabetic patients have an increased risk of long-term complications which are directly associated with duration of the diabetic state (Akerblom et al. 1997; Williams 1999). The natural history of autoimmune diabetes involves four phases of variable durations (Rewers and Klingensmith 1997). It is strongly asserted that every case of diabetes has both genetic and environmental causes (Rothman 2002).

There are indications that the destruction of insulin producing pancreatic beta cells may result from a series of unfavourable interactions between environmental factors over a limited period in genetically susceptible individuals. Having the knowledge that no causal links between any of the putative environmental factors and the initiation of the autoimmune process of the pancreatic beta cell have yet been identified unequivocally; it is apparent that a range of mechanisms of pancreatic beta cell destruction are operative in type 1 autoimmune diabetes (Kawasaki et al. 2004). A better definition of the nature of the environmental triggering factors, the target autoantigens and the effector mechanisms required for the autoimmune process will broaden our understanding of the disease aetiopathogenesis (Bach 1994).

It was therefore pivotal to embark on identifying the changes that lead to the initiation of insulin secreting pancreatic beta cell (β-cells) destruction, to not only minimise or stop its progression, but also to prevent the initiation of beta-cell destruction. The present review attempts to examine main controversial issues linked to the role of bovine milk proteins (BMP) in provoking the pancreatic autoimmune process in children, to explain how evident the relationship is, and what remains to be done to control it.
1.2 Autoimmune diseases: General background

Differentiation between self and non-self determinants enables the body to establish a must self-tolerance, a specific immunological non-reactivity to an antigen resulting from a previous exposure to the same antigen, mechanisms. The breakdown of these mechanisms triggers the initiation of an immune response against self-components. In autoimmune diseases, products of the immune system, antibodies and effector T cells, can cause damage to the self (Roitt et al. 2001; Ghaffar 2004).

Autoimmune diseases can be classified according to the organ or tissue involved into an organ-specific or a non-organ-specific disease. The immune response in the former category is directed against certain antigen(s) associated with the damaged target organ, for example Hashimoto’s thyroiditis, primary myxoedema, pernicious anaemia, Addison’s disease, and type 1 autoimmune diabetes. The immune response in the latter category is directed against antigens not associated with the target organ, for example ulcerative colitis, rheumatoid arthritis, anti-phospholipids syndrome, discoid and systemic lupus erythematosus (Roitt et al. 2001; Ghaffar 2004).

Human and animal studies have demonstrated the importance of genetic susceptibility in the development of the autoimmune disease particularly human leucocyte antigen (HLA) genotypes (B, B27, DR2, DR3, DR4, DR5) (Ghaffar 2004). Although the precise aetiopathogenesis of the autoimmune diseases remains unknown, it has been suggested that a number of factors are involved in the aetiology of the autoimmune disease including sequestered antigen, a void of autoreactive T cell clones, loss of suppressor cells, and cross reactive antigens (Roitt et al. 2001; Ghaffar 2004).
1.2.1 Type 1 autoimmune diabetes

1.2.1.1 Definition

The concept of type 1 diabetes as an immune-mediated disease developed rapidly in the mid-1970s (Gale 2001). Based on the disease aetiological heterogeneity and given that not all persons with T1ADM are children at onset and not all are insulin-dependent or having an immune-mediated form of the disease, the American Expert Committee on the Diagnosis and Classification of Diabetes Mellitus has recommended a new aetiological diagnostic criterion.

The first category of this four-category classification represents type 1 (insulin-dependent) diabetes, which is further divided into two subgroups: “type 1A” diabetes for immune-mediated diabetes, and ‘type IB’ for non-immune diabetes with severe insulin deficiency. The second category represents type 2 (non-insulin-dependent) diabetes ‘type 2 diabetes’. This category includes idiopathic types of diabetes with insulin resistance and without severe insulin deficiency or significant loss of β cells. The third category stands for gestational diabetes. Finally, a category for individuals with specific genetic syndromes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2002) such as Maturity Onset Diabetes of Youth-MODY (Winter et al. 1987).
The aetiology of autoimmune diabetes as a form of diabetes for immune-mediated diabetes is unknown. What is known however, is that the disease is a degenerative, multifactorial, complex, chronic, metabolic, slowly progressive, polygenic, organ-specific, inflammatory, autoimmune-mediated disorder induced by activated autoreactive T cells (Van Vliet et al. 1989; Roep et al. 1990; Thorsby and Ronningen 1992; Bach 1994; Davies et al. 1994; Neophytou et al. 1996; Wilder 1998; Roep et al. 1999; Mordes et al. 2004; Ogasawara et al. 2004; von Boehmer 2004; Nakayama et al. 2005), and characterised by genetically determined susceptibility (Heward and Gough 1997; Rich and Concannon 2002).

The occurrence of autoimmune markers (Kimpimaki et al. 2001), selective and aggressive destruction of insulin-producing pancreatic β-cells (Winer et al. 2003), the reported progress to glucose intolerance and chronic hyperglycaemia, and significant decrease in insulin receptors leads to insulin resistance, or “exceptionally” it happens due to the presence of receptor antibodies (McCarty et al. 1996).

1.2.1.2 A conflicting nomenclature

The massive amount of data generated worldwide on diabetes is described in many amorphous and often contradictory ways. To avoid the inevitable overlap between the published work of various research groups as well as to avoid arriving at conflicting nomenclatures, for example type 1 diabetes, type 1 (insulin-dependent) diabetes mellitus and juvenile diabetes. The term type 1 autoimmune diabetes mellitus (T1ADM) will be used throughout the present study to denote other popular ways of naming the disease, (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2002).
1.2.1.3 Phases

It is well established that the natural history of type 1 autoimmune diabetes involves four phases (Rewers and Klingensmith 1997). Firstly, the pancreatic β-cell pre-clinical slowly progressive prodromal phase characterised by selective destruction of insulin-producing pancreatic islet β-cells asymptotically until around 90% of the β-cells are destroyed through T-cell infiltrate accumulating around the islet cells and subsequently inside these cells leading to invasive ‘insulitis’ (Saukkonen et al. 1994; Levy-Marchal et al. 1995b; Simone and Eisenbarth 1996; Akerblom et al. 1997; Imagawa et al. 2000; Winer et al. 2003).

The first stage exists for up to 13 years, but it may be concluded in a few months (Plotinick and Hinderson 2001), even in utero, during which autoimmune and metabolic changes can be detected (Leslie and Elliot 1994; Knip 1997). Secondly, the clinical diabetes onset phase. Thirdly, the transient remission phase, and finally, the established diabetes phase (Rewers and Klingensmith 1997; Rewers et al. 2005). The presentation of clinical diabetes at variable ages may reflect various disease progression rates rather than different periods of exposure to possible critical environmental events (Otonkoski et al. 2000).
1.3 Facts and figures

Type 1 autoimmune diabetes has a worldwide distribution with substantial variation (Karvonen et al. 2000). Approximately 124 million individuals worldwide have diabetes, a minimum of three million have T1ADM, and the majority are children (International Diabetes Federation 2003). The global increase in the disease occurrence seems to affect both at low (Hungary) and at high (Sweden) risk populations (Soltesz et al. 1994) throughout the world and marked by obvious variations (Karvonen et al. 2000). The relative increase is predominantly higher in populations with lower occurrence (Bingley and Gale 1989; Handelsman and Jackson 1999; Onkamo et al. 1999). A Finnish occurrence trend analysis (1965-1996) study showed an absolute 0.67 annual increase in the disease occurrence (Tuomilehto et al. 1999), the overall increase in the disease occurrence calculated from pooled data based on 37 studies compiled in 27 countries from 1960 to 1996 was three percent per year (95% CI: 2.6; 3.3, P=0.0001) (Onkamo et al. 1999).

The occurrence of T1ADM will continue to increase above 30/100,000/year in several other populations (Onkamo et al. 1999). The increase in the occurrence of the disease in Finland (1987-1996) at diagnosis was the highest in children aged one to four years (Tuomilehto et al. 1999); while, the worldwide age-specific disease occurrence rate has increased with age, the highest was among 10-14 years-old children (P<0.0001) (Karvonen et al. 2000). Whether that trend in disease occurrence indicates a change in the age at disease onset instead of a definite increase in prevalence requires further investigation (Onkamo et al. 1999).
Globally, the Diabetes Mondiale (DiabMond) Study Project (Karvonen et al. 2000) has demonstrated a more than 350-fold variation in age adjusted disease occurrence rates; it has ranged from 0.1/10,000/year in Zuny, China to 36.8/10,000/year in Finland. The vast majority of Asian populations have a very low age-adjusted disease occurrence rate (<1/100,000/year), whereas an intermediate disease occurrence rate was observed in North African populations (5-9.9/100,000/year).

In Europe, the occurrence rate has ranged from 5.7/100,000/year in Latvia; 19.9/100,000/year in Norway; and 26.9/100,000/year in Sweden to approximately 37/100,000/year in both Sardinia and Finland. While the populations of North America have a high disease occurrence rate (10-19.99/100,000/year), the occurrence rate among South American populations has ranged from less than one to 9.99/100,000/year. The occurrence rate of the disease in the majority of Central America and West Indies populations has ranged from one to 9.9/100,000/year, whereas in Oceania, the age-adjusted occurrence rate has ranged from 14.5/100,000/year in New South Wales, Australia to 21.9/100,000/year in Canterbury, New Zealand (Karvonen et al. 2000).

Type 1 autoimmune diabetes is prevalent in the Middle East and estimates indicate that a minimum of 100,000 individuals have the disease (Amos et al. 1997a). The estimated annual disease occurrence was approximately 6000 with some 3000 being children below 15 years. In Jordan (1997), approximately 5200 Jordanians had the disease with 700 being children below 15 years old (Green 1997). The disease occurrence in Jordanian children aged 0-14 years is among the lowest in the region and has remained relatively unchanged.
Although the occurrence of the disease in Jordan has slightly increased from 2.8 per 100,000 in 1992 to 3.6 per 100,000/year in 1996 (Ajlouni et al. 1999), it remains among the lowest in its region and matches that of the Arab population who live in neighbouring Israel (2.9/100,000/year) (Shamis et al. 1997). Marked increase in the disease occurrence of T1ADM is occurring among the Jewish population with an exceptional increase in Yemenite Jews (18.5/100,000/year) compared to the overall occurrence rate in the Jewish population (5.7/100,000/year) (Shamis et al. 1997). The disease occurrence has also increased substantially in Kuwaitis. It has increased from 3.95/100,000/year (1980-1981) (Taha et al. 1983) to 14.4/100,000/year (1992-1993) (Shaltout et al. 1995).

An obvious variation in the disease occurrence rate exists between the northern and southern hemispheres with a positive polar equator gradient. No country below the equator has an occurrence more than (15/100,000/year) (Karvonen et al. 1993). The highest disease occurrence was observed in Caucasian people, and the lowest was observed in Asia and South America. Except in Nordic countries where certain exceptions from the overall disease occurrence pattern exist; Finland and Sardinia have the highest disease occurrence rates and are characterised with a unique genetic background, dissimilar environments, and are all geographically far from each other (Karvonen et al. 2000). European countries have shown the largest difference in the occurrence rates of the disease (Karvonen et al. 1993; Thorsdottir et al. 2000).

Considerable variation was also observed between the four countries around the Baltic sea (Padaiga et al. 1997). Although the disease occurrence rate within-country vary in Nordic countries, United Kingdom, America (Reunanen et al. 1988), Italy, New Zealand and China, dissimilarity within populations was not often reported. Possible underestimation of the occurrence of the disease in populations (i.e. Asia) with low disease occurrence necessitate careful interpretation of these worldwide variations in the occurrence rate of the disease (Karvonen et al. 2000).
The increase in the disease occurrence is not restricted to a particular ethnic group (Onkamo et al. 1999). Worldwide geographic variation in disease occurrence rates indicates that the distribution of ethnic groups and the degree of difference in genetic susceptibility exists between different races. For example, Blacks and Mongoloids have a lower disease occurrence compared to that of Caucasian populations (Reunanen et al. 1988). While European populations have the highest disease occurrence rate, it has been reported that tropical and nontropical populations have a relatively high disease occurrence rate marked with a relatively wider risk gradient among non-European ethnic groups (Karvonen et al. 2000).

Furthermore, it has been demonstrated that gender variations exist between populations. Populations with low disease occurrence rate have a female predominance (Rewers et al. 1988), whereas populations with high disease occurrence rate have a male predominance (Padaiga et al. 1997). In addition, the occurrence rate of diabetes follows an inverse association with mean annual temperatures. Seasonal variation of the disease occurrence in Europe follows a sinusoidal model in both sexes and all childhood age groups, with a peak in winter (Levy-Marchal et al. 1995a). Worldwide variation in the occurrence rates of T1ADM among and within major ethnic and racial groups in different populations signifies an important interplay between genetic susceptibility and environmental factors in the initiation and development of the disease (Karvonen et al. 2000).
1.4 The aetiopathogenesis of autoimmune diabetes

1.4.1 Who is more prone to have the disease?

The clustering of the disease in certain families as well as twin studies have demonstrated that genetic background plays a significant role in the occurrence of T1ADM (Heward and Gough 1997). A genome-wide linkage screen for human T1ADM susceptibility genes has identified more than 18 genomic regions that may harbour susceptibilities linkage for the disease. The polygenic inheritance nature of the disease reflects a genetic heterogeneity of T1ADM in diverse ethnicities (Davies et al. 1994).

Depending on the HLA DQ genotype in humans, the risk of developing T1ADM varies by a minimum of a thousand fold (Nakayama et al. 2005). Human leucocyte antigen genes class II region encoded within the major histocompatibility complex (MHC) on the short arm of chromosome 6p21 (referred to as IDDM1) comprises the major locus conferring disease susceptibility. An association with the insulin gene region on chromosome 11p15 (referred to as IDDM2) has also been proposed (Davies et al. 1994; Tosi et al. 1994). In addition to HLA gene region, it has been demonstrated that other genes on different chromosomes are implicated in susceptibility to have T1ADM. For instance, vitamin D receptor gene polymorphism (Motohashi et al. 2003) and susceptibility locus on chromosome 8q24 (Sale et al. 2002) have been implicated in susceptibility to T1ADM. Therefore, they may play a role in predicting the disease in individuals at high-risk.
The MHC class II includes at least three loci: HLA-DP, DQ and DR alleles, which comprise the major locus conferring disease susceptibility (Tosi et al. 1994). It appears that while more than a half of the genetic susceptibility to T1ADM is related to the HLA gene, the remaining portion is non-HLA associated. Ninety percent of patients with autoimmune diabetes have both or either DR3 and DR4 alleles (Reece et al. 1991). Although the association of particular combinations of DQ (A1 and B1) genes among Caucasoid, Black and Orientals has indicated that the greatest susceptibility is in the HLA DQ molecules (Thorsby and Ronningen 1992), a complete HLA DQ genotype should be determined to estimate the maximum risk for developing the disease (Tosi et al. 1994).

Sequence analysis of polymorphic heterodimeric HLA-DQ gene products has suggested that variation may also account for changes in the risk of developing T1ADM. For example, while an amino acid variation at position 57 on the DQ beta chain and non-aspartic acid was associated with a significant increase in risk of developing the disease, those having an aspartic acid residue in the same position were related to disease resistance. The presence of non-aspartic DQB1 and arginine DQA1 alleles has exhibited the highest risk of developing the disease. On the other hand, heterozygosis at DQB1 position 57 or DQA1 position 52 was adequate to negate the significance for association with developing T1ADM (Tosi et al. 1994).
Individuals who carry the HLA-DQB1*02/0302 risk genotype have a high increased genetic risk of developing T1ADM; whereas individuals who carry the HLA-DQB1*0302/x (x = other than *02, *0301, or *0602) have a moderate risk (Kimpimaki et al. 2002). A highly significant geographic variation in the risk HLA-DQ genotypes associated with T1ADM in five different populations with low, moderate, or high-risk HLA-DQ genotypes may explain the 30 fold hypothesised variation in the occurrence of autoimmune diabetes across ethnic groups as well as across populations (Dorman et al. 1990; Jacobs et al. 1992; Ilonen et al. 2000; Kukko et al. 2005). Correlating these variations with various HLA-DQ alleles particular function generate appealing insights into the potential mechanisms triggering off T1ADM (Moustakas and Papadopoulos 2002).

Emerging findings on gene and cell replacement therapy may constitute a novel approach for the treatment of T1ADM. It has recently been demonstrated that delivering a pancreatic and duodenal homeobox gene 1 (PDX-1), a known active gene in the development of insulin-producing pancreatic beta cells, to both foetal progenitor liver cells (Zalzman et al. 2003) and adult human, non-insulin producing, liver cells (Sapir et al. 2005) has induced a comprehensive developmental shift of these cells into functional insulin-secreting cells. High blood glucose levels of diabetic immunodeficient mice, recipients of the genetically engineered liver cells, were regulated for a prolonged period of time (Zalzman et al. 2003; Sapir et al. 2005).

Although the mechanisms of disease inheritance remain controversial (Reece et al. 1991), progress in the ability to identify persons at high risk has led to an ongoing worldwide intensive and extensive research. Consequently, Type 1 Diabetes Genetics Consortium (T1DGC) has been established to orchestrate the ongoing international endeavour to identify genes that designate an individual’s risk of type 1 diabetes (T1DGC 2003).
1.4.2 Autoantigens cross-reactivity

In view of host immune responses, it is increasingly evident that cellular response to autoantigens cross-reactivity may take a more significant part in disease aetiopathogenesis than was formerly anticipated. Molecular mimicry between host and foreign environmental factors are to a certain extent a frequent episode, and most likely take place much more frequently than once indicated (Visvanathan and Zabriskie 2000).

A significant body of evidence indicates that an invading foreign protein has an amino acid homology comparable to that in a host might initiate a breaking tolerance to self-antigens. Therefore, a viral, bacterial, or food peptide similar enough to a human peptide can cause an autoimmune reaction. For example, bovine serum albumin (BSA) and islet cell proteins ICA 69/p69; β-lactoglobulin (BLG) and retinolbinding protein; β-casein and GLUT-2 glucose transporters; conjoint autoantigenicity of proinsulin (amino acids 24-36) and GAD65 (amino acid 506-518) (Rudy et al. 1995; Narendran et al. 2003) have been anticipated to provide a molecular base to explain the initiation of cross-reactive immune responses directed not only at the invading foreign protein, but also toward self-proteins (Cavallo et al. 1996; Vaarala et al. 1998; Atkinson and Eisenbarth 2001). However, to accurately institute the relevance of molecular mimicry in naturally occurring human disease is, yet the most complicated phase (Oldstone 2000).
1.4.3 Invasion of autoreactive lymphocytes

Increased immunity to BMP is not a disease-specific immunity; it reflects associated HLA haplotypes genetic susceptibility to increased immunity to dietary proteins that is associated with the HLA-A1-B8-DR3-DQ2 (A1*0501, B1*0201) haplotype (Harrison and Honeyman 1999). The exact mechanism by which the HLA-DQ molecules and other class II molecules are directly involved in the initiation of β-cell destruction remains unclear. Nonetheless, it seems likely that β-cell destruction involves antigens peptide fragments presentation by the class II gene encode heterodimeric proteins expressed on the surface of antigen-presenting cells such as dendritic cells, lymphocytes, monocytes/macrophages to CD+$^+$ T-helper cells (Thorsby and Ronningen 1992; Moustakas and Papadopoulos 2002; von Boehmer 2004).

The detection of particular β-cell derived peptides by autoreactive CD8+$^+$ T helper cells may well be a key episode in initiating the autoimmune process of the disease (Thorsby and Ronningen 1992; Ogasawara et al. 2004; von Boehmer 2004). Activated islet-specific T cell clones have been found to transfer diabetes to non-obese diabetic (NOD) mice demonstrating that activation of autoreactive T cell is vital for the initiation of autoimmune diabetes (Peterson et al. 1994; Wucherpfenning and Strominger 1995; Peterson and Haskins 1996). It has been found also that the mutation of proinsulin transgene (residue 16 on the insulin B chain was replaced by alanine) has abrogated the T-cell stimulation of the major insulin autoreactive NOD T-cell clones (Abiru et al. 2000). Vitamin D compounds may suppress the activation process by binding to vitamin D receptor (Motohashi et al. 2003), nevertheless the activation process might be triggered by a number of mechanisms such as molecular mimicry, viral or bacterial superantigens; release of autoantigens during inflammation; and bystander activation (Fujinami 2001).
Innate immune Natural killer (NK) cells express neither T-cell nor B-cell antigen receptors; most surface antigens detectable on NK cells by monoclonal antibodies are shared with T cells (Roitt et al. 2001). The balance between signals transmitted by activating receptors regulates the function of the NK cells (Cerwenka and Lanier 2001). The identification of receptors of NK cells has enhanced our understanding of the role of NK cells in immune responses (Yokoyama and Plougastel 2003). Studies on NOD mice have demonstrated that CD8$^+$ T infiltrating the pancreas express NKG2D, an activating receptor on CD8$^+$ T cells and NK cells; antibodies blocking NKG2D during the prediabetic stage can completely prevent the disease progression through impairing the expansion and function of autoreactive CD8$^+$ T cells (Ogasawara et al. 2004). It has been also found that the suppressor CD25$^+$ and CD4$^+$ regulatory T cells can suppress autoimmune disease in vivo (Tarbell et al. 2004).

A small number of CD25$^+$ and CD4$^+$ regulatory T cells can reverse diabetes after the onset of disease in animals (Tang et al. 2004). This may harness a new approach for autoimmune diabetes cellular immunotherapy (Tang et al. 2004; Tarbell et al. 2004). Better understanding of the mechanisms policing the migration and subsequent invasion of autoreactive lymphocytes and other leukocytes from the blood into the pancreas causing insulitis and beta cell destruction will offer a better understanding of the aetiopathogenesis of autoimmune diabetes (Yang et al. 1996).
1.4.4 Circulating autoantibodies: Early markers

Type 1 autoimmune diabetes is characterised by the early presence of autoantibodies against islet β-cell derived proteins (Narendran et al. 2003). Cytoplasmic islet cell antibodies (ICAs), a heterogeneous group of islet autoantibodies (Eisenbarth et al. 1992; Zanone et al. 2003), are deemed to a great extent as a significant humoral marker for the prediction of a β-cells autoimmune process, an early indicator of T1ADM (Bingley et al. 1994). In addition, a wide range of circulating autoantibodies to islet autoantigens are anticipated as possible pancreatic autoimmunity markers of both human and NOD mouse.

The main autoantigens implicated in the disease aetiology include insulin and proinsulin (Palmer et al. 1983; Ziegler et al. 1989; Narendran et al. 2003; Nakayama et al. 2005), glutamic acid decarboxylase 65 (GAD65) (Baekkeskov et al. 1990; Tisch et al. 1993; Tisch et al. 2001; Zanone et al. 2003), tyrosine phosphate related enzyme protein IA-2 (ICA512/IA-2A) (Lan et al. 1994; Kawasaki et al. 1996; Wasmeier and Hutton 1996; Zanone et al. 2003) and heat shock protein 65 (Elias et al. 1990; Elias et al. 1991; Birk et al. 1996). Other alternatives include increased levels of local nitric oxide, oxygen radicals, and certain cytokines (Kolb et al. 1995).

In view of the number of autoantibodies positivity, siblings of children newly diagnosed with T1ADM can be categorised into four stages: “no prediabetes”; “early prediabetes”, “advanced prediabetes” and “late prediabetes” for no autoantibodies, one, two and more than three autoantibodies respectively (Mrena et al. 1999). It has been demonstrated that insulin autoantibodies (IAA) are the first to appear in diabetic children less than three years of age (Yu et al. 2000) whereas IA-2A antibodies are the most specific predictor, islet cell autoantibodies (ICA) and IAA are the most sensitive predictors for T1ADM (Kukko et al. 2005).
While GAD65 autoantibodies and IA-2A autoantibodies were considered as the main markers for the prediction of T1ADM (Zanone et al. 2003), the presence of autoantibodies for all possible autoantigens, ICA, IAA, GAD65 and IA-2A was considered as a stronger indicator of a late sign of an in-progress β-cells autoimmune process (Kimpimaki et al. 2001). Nevertheless, GAD65, and IA-2A had the highest predictive value for T1ADM (Kukko et al. 2005).

Increased islet autoantibody levels among infants most likely exhibit a sign of de novo production of autoantibodies associated with T1ADM (Ziegler et al. 1999; Hamalainen et al. 2000). The detection of de novo produced islet autoantibodies prior to one year of age is vital as offspring have an extremely high risk of developing the disease. Children born to mothers with T1ADM have maternally acquired antibodies that continue for nine months at least after birth; antibodies to GAD65 were nearly transient maternally acquired at nine months of age, whereas IAA were often non-maternally acquired at nine months of age (Naserke et al. 2001).

Foetal exposure of the offspring of mothers with diabetes to islet autoantibodies may be protective to potential islet autoimmunity and, subsequently diabetes (Koczwara et al. 2004). Although most cord blood autoantibodies seem to be maternally acquired, the presence of autoantibodies in infants cord blood has no predictive value of future development of islet β-cell autoimmunity (Stanley et al. 2004).
Replace testing for ICAs, as a primary screening test, by testing for either IA-2A/GAD65 or IAAs/GAD65 if combined with second line tests for ICAs and/or IAAs was found to be more sensitive. Therefore, the sample of population required will be reduced to half of that needed if tested for ICAs only (Bingley et al. 1999). The increased levels of antibodies to ICAs, insulin, GAD65, and IA-2A identified more frequently at two years of age with no particular order in the occurrence of positive autoantibodies tested have indicated that the pancreatic autoimmune process starts very early in life among genetically susceptible children. As such, more attention to this age-group may be necessary in screening stages (Roll et al. 1996).

It has been found that of six children tested positive for all four-islet autoantibodies, three children developed clinical diabetes before five years of age. These findings demonstrate an increased risk for a probable future development of the disease among genetically susceptible children, and therefore, exhibit a high predictive value (Roll et al. 1996). A Finnish prospective study has proposed that while ICAs seem to be more specific, IAAs are characterised by high sensitivity, early emergence, and high occurrence of transit antibody positivity for the screening process among children genetically susceptible to autoimmune diabetes during the first two years of age (Komulainen et al. 1999; Paronen et al. 2000b; Kimpimaki et al. 2002). Autoantibodies positivity to either IA-2A or GAD65 denotes a highly sensitive marker for β-cell autoimmunity in the paediatric age group. The perseverance of IA-2A confers a sign for residual function of β-cell, hence providing clinical and predictive significance to these autoantibodies (Zanone et al. 2003).
A study on dizygotic and monozygotic non-diabetic twins and non-twins compared to diabetic patient siblings and controls has indicated that only 5.9% of the controls (P<0.0001), 10.7% of the non-twin siblings (P<0.0001) and 20% of the dizygotic twin siblings (P<0.05) compared to 41.5% of the monozygotic twin siblings exhibited ICAs, IAA, GAD65, and IA-2A. Monozygotic twin siblings have shown multiple autoantibodies positivity more frequently than the dizygotic twin siblings. Survival analysis demonstrated that monozygotic twin siblings with HLA-DQ8/DQ2 genotype were more likely to express positive autoantibodies than monozygotic twin siblings without this genotype (64.2%; 95% CI 32.5-96, and 23.5% CI 7-40, respectively) at ten years discordance (P<0.05). These findings suggest a possible predominance of a genetic susceptibility in islet β-cell autoimmune process (Redondo et al. 1999).
1.4.5  ABO and Rh blood groups: Signs of protective effect

A specific complex of antigens present on red blood cells genetically determines blood group characteristics. Immunological characteristics determine and classify the differentiation of blood by type (Watkins and Morgan 1959; Morgan and Watkins 2000). A wide range of blood group systems is identified. The ABO blood groups system, first introduced in 1901, is the most used blood group system. Hitherto, more than 25 major blood group systems, a minimum of 270 red cell phenotypes and more than six hundred red cell membrane antigens are recognised (Green 1989; Garratty et al. 2000; Hughes-Jones and Gardner 2002).

Studies have demonstrated the implication of different blood group phenotypes in disease pathogenesis. The ABO and Rhesus [Rh (D)] blood groups have been implicated in increased susceptibility to certain diseases (Blackwell 1989), for instance *Helicobacter pylori* and the increased risk of having peptic ulcer (Alkout et al. 1997; Alkout et al. 2000), haemolytic uremic syndrome and *Escherichia coli* (Blackwell et al. 2002), elevated serum antibody titre levels to *vibrio cholera* (Swerdlow et al. 1994), carcinomas (Su et al. 2001) and infertility in women (Lurie et al. 1998).
The ABO blood group system is the most important and most common human alloantigen system in blood transfusion (Yamamoto et al. 1992). The genetic role in the determination of ABO and Rh (D) blood groups (Green 1989; Garratty et al. 2000; Hughes-Jones and Gardner 2002) exhibited extensive variation in the distribution of ABO and Rh (D)D blood groups in different populations (May and du Toit 1989; Wagner et al. 1995; Falusi et al. 2000; Chiaroni et al. 2004). The main ABO alleles that create familiar A, B, AB and O blood groups exhibit broad sequence heterogeneity and extensive sequence variation in both the coding and non-coding region of the gene, given that a minimum of 70 ABO alleles was reported (Yip 2002).

An increased risk of developing type 1 autoimmune diabetes due to maternal-child blood group incompatibility was established in Swedish diabetic children aged 14 years or less (Dahlquist and Kallen 1992). Studies on type 2 NIDDM (non-insulin-dependent diabetes mellitus) showed inconsistency in the distribution pattern of ABO and Rh (D) blood groups. While the distribution of B blood group was elevated among Pakistanis who have type 2 NIDDM (Qureshi and Bhatti 2003), the proportion of A, AB and Rh (D) positive blood groups were increased among Indians who have the disease (Sidhu et al. 1988), whereas no significant difference was established in Iranians with diabetes (Karamizadeh and Amirhakimi 1996). These differences in the frequency of ABO and Rh blood groups indicate racial and ethnic variations in persons indigenous to various parts of the world (May and du Toit 1989; Wagner et al. 1995; Falusi et al. 2000; Chiaroni et al. 2004).

Furthermore, a significant relationship was found for the prevalence of fast acetylator phenotype in diabetic adults having blood group B, given fast acetylator prevalence was significantly higher in diabetic children than adults and non-diabetics (Pontiroli et al. 1984). While no associations were found for MN, KIDD and Lewis blood group systems in both diabetic adults and diabetic children grouped according to insulin dose administered, it was statistically significant for the ABO blood group in patients receiving higher insulin doses (Kanazawa et al. 1983).
Numerous studies have challenged the importance of ABO blood group in the occurrence of various diseases. For instance, the increased binding of *Helicobacter pylori* to epithelial cells and increased risk of peptic ulcer (Alkout et al. 1997; Alkout et al. 2000), duodenal and gastric ulcers (Mentis et al. 1991), haemolytic uremic syndrome caused by *Escherichia coli* O157 (Blackwell et al. 2002), diarrhoea and heat-labile enterotoxin-producing *Escherichia coli* (Black et al.) and elevated serum antibody titre levels to *vibrio cholera* (Swerdlow et al. 1994) are associated with a higher proportion of people of O blood group phenotype.

The ABO histo-blood system includes three antigens (ABH). Unlike people of A, B and AB groups who can convert the H antigen into A or B antigens, people with O blood group having guanine (258 residue) deleted in the O gene, produce an inactive protein incapable of converting the H antigen (Yamamoto F 1990). The prevalence of *Helicobacter pylori* in non-secretor people with duodenal ulcer was significantly higher than that of non-secretor people who had gastric ulcer (Mentis et al. 1991). An association between secretor status and the susceptibility to develop type 1 autoimmune diabetes with elevated proportion of non-secretors among them was also demonstrated (Blackwell et al. 1987).

Given the heterogeneity in the glycoconjunctates expression associated with different histo-blood group ABO (Yamamoto F 1990; Yamamoto et al. 1992; Yamamoto et al. 1993), it was established that the secretor gene shows signs of a protective effect particularly in immunocompromised people (Blackwell 1989). Studying the secretor status of humans with type 1 autoimmune diabetes and viral infections particularly rotaviruses (Honeyman et al. 2000) and enteroviruses (Hiltunen et al. 1997; Salminen et al. 2004), raises some further important questions with regard to the aetiopathogenesis of the disease (Blackwell 1989).
1.4.6  *Maturity and architecture of gut system*

Few studies have been carried out on the gut mucosa of patients with T1ADM, nonetheless the role of the gut immune system has been implicated in the aetiopathogenesis of the disease (Westerholm-Ormio et al. 2003). Gut mucosa constitutes the first immunoregulatory barrier (Harrison and Honeyman 1999). Not fully matured gut mucosa is most probably temporarily permeable to intact BMP during the first months of age (Savilahti et al. 1988; Kuitunen et al. 1994).

Mononuclear cell mass accumulates in the islets of Langerhans and obliterated pancreatic β-cells. T-cell lines isolated from the islets of children newly diagnosed T1ADM have demonstrated a strong adherence to the endothelium of both the diabetic pancreas and to the mucosal lymphoid tissue. Cell lines of the pancreatic or control show no adherence to the vessels of healthy pancreas suggesting that lymphocytes derived from the mucosal lymphoid tissue may be involved in developing the disease (Hanninen et al. 1993).

Breast milk has growth factors and cytokines, for instance insulin (Arsenault and Menard 1984; Buts et al. 1997a; Marandi et al. 2001; Shamir et al. 2001) among other immunomodulatory factors which enhance the functional maturation of intestinal mucosal tissues (Harrison and Honeyman 1999). Studies on artificially reared rat models have demonstrated that bovine milk-based formulae is a primary factor provoking intestinal overgrowth and precocious maturation of particular intestinal functions (Dvorak et al. 2000) particularly leading to structural and enzymatic alterations (Yeh 1983).
Human breast milk contains a higher concentration of insulin compared to that of bovine milk (60.23 ± 41.05 and 16.32 ± 5.98 μU/ml) (Shehadeh et al. 2001). No matter how insulin is administered (i.e., systemically or orally), it can interact with intestinal mucosa producing significant physiological consequences on gastrointestinal tract growth (Shamir et al. 2001). However, insulin available naturally in milk is most likely to be much more active on the gut than free orally administered insulin (Buts et al. 1997b).

Changes in dietary ingredients, microbial flora (Sharma et al. 1995b; Sharma et al. 1995a), bacterial metabolites (Fontaine et al. 1996), and enteric viral infections (Hiltunen et al. 1997; Honeyman et al. 2000; Salminen et al. 2004) as well as interaction between these factors may significantly change the mucosal architecture, the mucosal adaptation (Sharma and Schumacher 1995), the epithelial cell production (Clark et al. 1981), and the regional number of the enteroendocrine cells (Sharma and Schumacher 1996). This may increase the gut permeability to possible foreign, dietary, and non-dietary antigens. The interaction of the gut immune system with oral antigens may either boost or suppress the autoimmune process that depends on the functional status of the gut immune tissue (Scott et al. 1996a; Harrison and Honeyman 1999).

It is implicated that intraepithelial T lymphocytes of the small intestine have the ability to mature and differentiate independently either with the presence or absence of the thymus; therefore, the intestinal-associated lymphoid tissue could mediate the effect of diabetogenic dietary factors (Lefrancois and Puddington 1995). Therefore, the stabilisation of the mucosal barrier may play an important role in the maintenance of the gut health (Kleessen et al. 2003).
Diabetes-prone BioBreeding (BBdp) rats exposed to oral diabetogenic diets and immunomodulators were able to regulate the expression of T1ADM in connection with the changes in the gut local cytokine balance (Scott et al. 2002), resolving whether impaired mucosal architecture as well as the functionality of the immune system predisposes to the development of type 1 diabetes or not will enhance our understanding on the aetiopathogenesis of autoimmune diabetes in humans (Harrison and Honeyman 1999).

1.4.6.1 Uptake of macromolecules

While it has been suggested that elevated antibodies in humans may reflect de-novo production of autoantibodies associated with T1ADM (Hamalainen et al. 2000), the detection of circulating antibodies to BSA in young rabbits born to immunised mothers compared to those born to unimmunised mothers has given evidence on passive transplacental immunization in animals (Kleinman et al. 1983). Less intact BSA and more fragments of BSA were recovered from orally immunised rats than from unimmunised rats which demonstrates the role immunisation had on enhancing the degradation of antigen (Pang et al. 1981).

Colostrum is essential to the growth and function of the intestine particularly just after birth as it provides immunological defence. It has been demonstrated that it stimulates both intestinal endocytotic and enzymatic capability, and therefore failure to administer colostrum on time may affect the mediation of intestinal macromolecules transmission as well as the activity of disaccharidases (Westrom et al. 1985; Jensen et al. 2001).
In vitro and in vivo animal studies have shown that very small amounts of intact and polypeptide fragments of BSA may transfer across the intestine mucosa into the systemic circulation of mature animals (Bloch et al. 1988). Animal studies have further demonstrated the presence of immunoreactive BSA, molecular characteristics approximated that of native BSA and porcine albumin in the circulation of young piglets was enhanced during the early invasive stage of viral gastroenteritis (Bloch et al. 1979; Keljo et al. 1987; Egberts et al. 1991). An increase in intestine permeability following viral enteritis seems to take place only in Peyer’s patch-free jejunum segments and therefore future immunological implications have to take the specialised function of Peyer’s patch regions of the small intestine into consideration (Keljo et al. 1985).

1.4.6.2 Breastfeeding or bottle-feeding

The hypothesis of an inverse correlation between the protective effect that breastfeeding may have on the development of T1ADM and the implication of the early introduction of BMP at early age with the disease aetiopathogenesis has undergone worldwide extensive investigation. Evidence on the protective effect that breastfeeding may have on the development of the disease and the avoidance of BMP in early infancy has come from retrospective studies (Mayer et al. 1988; Glatthaar et al. 1988.; Blom et al. 1991; Virtanen et al. 1991; Dahlquist et al. 1992; Virtanen et al. 1992; Verge et al. 1994; Gimeno and de Souza 1997; Kimpimaki et al. 2001). The only prospective clinical dietary intervention trial on humans has recently suggested a possible role that dietary intervention in infancy may have on the initiation of pancreatic autoimmunity in genetically susceptible infants (Akerblom et al. 1999; Akerblom et al. 2005).
Whole population ecological studies have indicated a positive relationship between the exposure to BMP and the development of T1ADM in 12 countries and further have demonstrated in another 18 countries that populations having the lowest occurrence of breastfeeding at three months of age experience the highest occurrence of the disease (Scott 1990). These findings were supported by a previous study of 12 different countries (Dahl-Jorgensen et al. 1991) as well as in nine regions within Italy (Fava et al. 1994).

Studies have indicated that independent of duration of breastfeeding, young age at introducing BMP is the most vital risk factor for the development of autoimmune diabetes (Kostraba et al. 1993; Virtanen et al. 1993; Verge et al. 1994); exposing infants before eight days of age to dietary proteins other than breastfeeding can be a risk factor for the development of the disease (OR 2.29; 95% CI 1.37–3.83) (Gimeno and de Souza 1997). A population-based-control study has ascertained that while increased risk of developing T1ADM was associated with early introduction of bovine milk products before three months of age, exclusive breastfeeding for more than three months of age was associated with lower risk (OR 0.66; 95% CI 0.45-0.97). Increased risk was associated with high dietary intake of BMP in the 12-month period preceding the appearance of the disease symptoms later in childhood (OR 1.84; 95%CI 1.12-3.00) (Verge et al. 1994).
Retrospective human studies were not able to demonstrate the protective effect of the duration of breastfeeding and the delay in the introduction of BMP may have on the development of the disease (Fort et al. 1986; Siemiatycki et al. 1989; Bodington et al. 1994; Norris et al. 1996; Meloni et al. 1997; Esfarjani et al. 2001). Meta-analysis studies were able to establish a weak relation between the protective effect of breastfeeding and age at introducing BMP (Gerstein 1994; Norris and Scott 1995). A critical analysis of the clinical literature (available up to 1994) that links breastfeeding and early consumption of BMP to the development of T1ADM has concluded that diabetic patients in case control studies were more likely breastfed for less than 3 months of age (OR 1.43; 95% CI 1.15–1.77), and exposed to bovine milk before the age of 4 months (OR 1.63; 95% CI 1.22–2.17 (Gerstein 1994). This was confirmed by data from a summary of 17 case-control studies (Norris and Scott 1995) which demonstrated that early exposure to breast-milk substitutes (OR 1.38; 95% CI 1.18–1.61) and bovine milk-based substitutes (OR 1.61; 95% CI 1.31–1.98) before three months of age have a moderate effect on the risk of the development of autoimmune diabetes.

No significant difference has been found for the proportion of genetically susceptible children and autoantibodies associated with exposure to BMP. Children with autoantibodies were breastfed for a relatively longer period of time than their controls (Norris et al. 1996). Regardless of the HLA genotypes (Hummel et al. 2000), the Australian AudDiab (Couper et al. 1999) and the German BABYDIAB (Hummel et al. 2000) study groups were not able to demonstrate that breastfeeding may have a protective effect on the development of the disease. Although Sardinia has one of the highest disease occurrence rates in Europe, and the population is at a high risk of developing the disease, a case-control study has argued that even the mainstream proportion of children with diabetes were breastfed. The risk of developing the disease amongst those who were not breastfed was insignificant (Meloni et al. 1997). A Swedish and Norwegian time-series study (1940-1980) has ascertained an inverse relationship between the duration of breastfeeding and the development of T1ADM (Borch-Johnsen et al. 1984).
Similarly, a cross-sectional study screening children from families with a history of T1ADM for beta-cell autoimmunity markers (IAA, GAD, and IA-2) has found no significant differences for the relation to exposure to BMP between children who have autoantibodies and their controls. Children with autoantibodies were breastfed for a longer duration than the controls (Norris et al. 1996). These findings were supported by a prospective study which has indicated that even in high-risk children (Relative risk: 0.91-1.09) no potential relation has been established between the duration of breastfeeding or exposure to BMP and the initiation of beta-cell autoimmune process (Couper et al. 1999).
1.5 Non-genetic determinants of the disease

The data indicate that while there is an important genetic component to T1ADM, (Heward and Gough 1997; Rich and Concannon 2002), different putative environmental factors may also play a major role in development of the disease. Twin studies (Verge et al. 1995; Petersen et al. 1997; Redondo et al. 1999; Redondo et al. 2001), migrant studies (Bodansky et al. 1992; Shamis et al. 1997), worldwide geographic variations (Karvonen et al. 2000; Atkinson and Eisenbarth 2001), interethnic and racial differences (Shamis et al. 1997; Karvonen et al. 2000), geographic variation in the risk genotypes for diabetes (Kukko et al. 2004), and disease occurrence transitory tendency have indicated that environmental factors operating in genetically susceptible individuals may play a crucial role over a limited period of time in the disease aetiopathogenesis (Elliot et al. 1996; Knip 1997).

Epidemiological studies have indicated a strong aetiological role of non-genetic risk determinates for T1ADM without discriminating between low (Hungarian) and high (Swedish) at risk populations (Soltesz et al. 1994). Certain viral infections (Yoon et al. 1979) and several dietary factors, particularly BMP, wheat and soy proteins are among the most putative environmental triggering agents in the initiation of a pancreatic β-cell destruction process (Akerblom and Knip 1998; Norris et al. 2003; Ziegler et al. 2003).
In addition, toxins, particularly n-nitroso compounds, vaccine administration, psychological stress (Atkinson and Eisenbarth 2001), age group, seasonal variation (Levy-Marchal et al. 1995b), geographic variation (Levy-Marchal et al. 2001), sex, maternal blood incompatibility, infectious diseases, high growth rates (Dahlquist 1994), social status, foetal and perinatal events (Soltesz et al. 1994), and overweight (Libman et al. 2003) are all non-genetic factors implicated in the aetiology of the disease. Furthermore, it has been indicated that vitamin D deficiency has a potential role in the aetiopathogenesis of autoimmune diabetes (Stene et al. 2000; Hypponen et al. 2001; Norris 2001) and that autoimmune process targeting nervous system structures surrounding insulin producing islet beta cells appear to be an integral early part of the development of the disease (Winer et al. 2003).

### 1.5.1 Implication of viral infections

Exemplifying a model for intrauterine virus-induced diabetes, congenital rubella syndrome is the only viral infection evidently linked to the disease (Ginsberg-Fellner et al. 1984). Nonetheless, since there is no evidence of increased frequency for humoral beta cell destruction in patients with congenital rubella syndrome, it has been proposed that the development of T1ADM in response to this infection may be triggered by a mechanism other than autoimmune mechanisms (Menser et al. 1974; Viskari et al. 2003). Although most of the evidence on the role of viral infections in the aetiopathogenesis of T1ADM in genetically susceptible children has come from epidemiological studies (Dahlquist 1991), a few prospective studies have shown a significant and specific potential association between both rotavirus (Honeyman et al. 2000) and enteroviruses (Hiltunen et al. 1997; Salminen et al. 2003; Salminen et al. 2004), and the initiation of the pancreatic autoimmune process.
Although there is a tendency for higher enterovirus infections among enterovirus positive participants than controls, it provides no evidence to support the view that enteroviral infection frequency is a possible risk factor for pancreatic autoimmunity among genetically at risk children. Further investigation is required to determine whether continuous or recurring viral infections occur more commonly among individuals with pancreatic autoimmunity or not (Graves et al. 2003).

1.5.2 Casein: A renowned diabetogen

Although 2.5 fold increased risk of developing autoimmune diabetes is associated with early exposure to solid foods, the evaluation of infants’ exposure to other foods in their diet has not been studied as extensively as BMP (Rewers and Norris 1996). Studies on BBdp rats (Elliot and Martin 1984; Scott et al. 1989; Hoofar et al. 1991; Schatz and Maclaren 1996) have showed subsequently that diabetes occurrence is highest in animals fed mainly on plant protein-based diet, mainly wheat and soy proteins.

It remains unknown how wheat proteins increase disease expression in genetically susceptible animals. Scott’s group has described and partially characterized food diabetogens in wheat protein and soy protein, which might be more antigenic than BMP. These food antigens are linked to MHC class I expression on β cells, reduced islet area, β-cell mass and a preponderance of Th1 cytokines in pancreas (Scott 2003). In the period prior to the development of significant pancreas inflammation in BBdp rats, a number of diet-modifiable changes have taken place in gastrointestinal tract inflammatory mediators and permeability (Hurley 1993; Scott 2003).
Nordic countries have a similar genetic background as well as similar breastfeeding practices. Although Icelanders are amongst the highest in the world-consuming bovine milk per capita (International Dairy Federation 1993; Elliot et al. 1999), the occurrence of autoimmune diabetes in Iceland is less than 50% of that in other Nordic countries. No significant difference has been found between exclusively breast-feed diabetic children and newly diagnosed diabetic children who received bovine milk-based formula and the control group. The frequency and extent of breastfeeding were comparable. Lower T1ADM occurrence might be explained by the significantly lower levels of A1 and B β-casein variants of Icelandic cow milk compared to that in other Scandinavian countries (Thorsdottir et al. 2000).

Similarly, Elliot found that all analysed Icelandic milk samples have lower β-casein fraction A\textsuperscript{1} and B β-casein than other milk samples from Scandinavia. Per capita A\textsuperscript{1} and B β-casein and autoimmune diabetes occurrence are significantly correlated (Elliot et al. 1999). Unlike other Scandinavian cattle breeding, Icelandic cattle have been isolated for about 1100 years leading to less frequent gene coding for A\textsuperscript{1} and B β-casein (Lien et al. 1999).

Studies on NOD mice have demonstrated that whole casein diabetogenicity is produced from β-casein A\textsuperscript{1} A\textsuperscript{1} cows; nonetheless, casein produced from β-casein A\textsuperscript{2} A\textsuperscript{2} β-casein cows did not. This effect may be due to the presence of a bioactive β-Casomorphin-7 peptide (BCM-7) that has opioid characteristics including a dramatic inhibitory effect on immune cell function in both susceptible human and animals. BCM-7 is released from the β-casein variant (A\textsuperscript{1} and B) with a histidine at position 67. However, it cannot be released from of the β-casein variant with a proline at position 67 (Elliot 1989; Elliot et al. 1997; Elliot et al. 1999).
Unlike β-casein A\textsuperscript{1} 60-67, bovine β-casein A\textsuperscript{2} and human β-casein contain a proline amino acid at a corresponding position in their sequence. It is likely due to this proline peptide that an equivalent to BCM-7 cannot be formed upon digestion of human β-casein (Elliot et al. 1997). Specific proliferation of T lymphocytes with bovine casein in persons with T1ADM may account for the response of pancreatic β-cells cellular and humoral immune response to β-casein (Cavallo et al. 1996). This explains the specificity of the connection between the occurrence of diabetes and the consumption of a number of β-casein variants (Elliot et al. 1999). However, casein hydrolysate-based bovine milk formula was found to be highly effective in preventing autoimmune diabetes in NOD mice (Karges et al. 1997).

Human preventive studies have shown that the elimination of dietary gluten for six months does not decrease autoantibody titres associated with autoimmune diabetes although it may have an advantageous outcome on the protection of β–cells in diabetic patients (Hummel et al. 2002; Pastore et al. 2003). Whether casein hydrolysate-based bovine milk formula is effective in preventing T1ADM in animals or not is still controversial. While an animal prevention study has found that casein hydrolysate-based bovine milk formula was highly effective in preventing autoimmune diabetes in NOD mice (Karges et al. 1997), another animal study on BBdp rats has concluded that protein free-based diets do not protect rats from developing diabetes (P<0.05). Rats fed on free amino acid-based diet showed a slight delay of developing the disease. Nevertheless, that concurred with a reduction in weight gain compared with rats fed on standard diet (Simonson et al. 2002).
A leading prospective study of T1ADM at high-risk population (BABYDIAB) has found that feeding newborn children with gluten-containing foods prior to three months of age was accompanied with a significant increase in islet autoantibodies risk (Adjusted hazard ratio 4.0; 95% CI 1.4-11.5; P=0.01) compared to those exclusively breast-fed until three months of age. The duration of exclusive breastfeeding or shortened total breastfeeding did not increase the risk of developing islet autoantibodies significantly (Ziegler et al. 2003).

The American Diabetes Autoimmune Study in the Young (DAISY), established in 1994 to investigate newborns with and without a family history of type 1 DM, has concluded that apart from the type of cereals introduced, the risk of islet autoimmunity was higher in infants aged zero to three months and those aged seven months when first given cereals than those aged between four to six months. This has pointed to a possible window of the introduction of dietary cereals in early infancy where primary exposure may add to the risk of developing islet autoimmunity (Norris et al. 2003). Nevertheless, the German BABYDIAB prospective study of high-risk population established in 1989, and the DAISY studies were not able to offer adequate proof to propose that feeding an infant on cereals triggers diabetes. The link between early infant diet and the development of autoantibodies produced against the pancreatic β-islet cells remains controversial (Atkinson and Gale 2003).
1.5.3 Bovine serum albumin: A strong candidate

A number of factors have prompted researchers to investigate bovine milk proteins particularly bovine serum albumin and bovine insulin as a putative environmental factors implicated in the destruction of the pancreatic beta cell. These factors include: first, the exposure of children to bovine-based formulae as their first foreign dietary supplement; second, extreme increase in the disease occurrence observed among children under 15 years old at diagnosis (Onkamo et al. 1999; Gale 2002); and finally, increased antibody titre levels produced against BMP in diabetic children compared to that produced in the controls have placed BMP particularly BSA under intensive and extensive investigation (Karjalainen et al. 1992b; Saukkonen et al. 1994; Levy-Marchal et al. 1995a).

Albumin is the most abundant protein in the blood circulatory system. Bovine serum albumin constitutes only one per cent of whole bovine milk (Carter and Ho 1994; National Health and Medical Research Council 2003). The predominantly alpha-helical polypeptide globin structure of BSA (Carter et al. 1989) consists of 607 amino acids (66430.3 Daltons) and seventeen disulphide bridges which form nine loops creating three domains (Feldhoff and Peters 1975; McLachlan and Walker 1977; Carter et al. 1989; Hirayama et al. 1990; Carter and Ho 1994). Bovine serum albumin amino acid sequence of peptide (1-24) has a high degree of similarity with that of human serum albumin. They only differ at four amino acid sites (2 [Threonine-Alanine], 6 [Isoleucine-Valine], 18 [Histidine-Asparagine] and 21 [Glycine-Alanine] respectively) (Bradshaw and Peters 1969).
1.5.3.1 *Humoral immunity*

Humoral immunity has been widely implicated in the initiation of the pancreatic β-cells autoimmune process. Studies have suggested that BMP may constitute putative triggering factors of the autoimmune response, attributing to antibody production and succeeding the destruction of β-cells in newly diagnosed children with the disease (Karjalainen et al. 1992a; Savilahti et al. 1993; Saukkonen et al. 1994; Levy-Marchal et al. 1995a; Saukkonen et al. 1995; Ahmed et al. 1997). The most direct evidence on humoral immunity to BMP has come from a Finnish family retrospective study, which has demonstrated that IgG antibodies to BSA, but not other BMP, were elevated in all newly diagnosed children. Most of the antibodies were age dependent and specific to the ABBOS epitope (pre-BSA position 153-169 peptide).

Since ABBOS shares an epitope with p69 β-cell surface protein may induce a cross-reactive immune response, ABBOS peptide has been implicated as a possible triggering factor of the autoimmune process (Karjalainen et al. 1992a). Studies have demonstrated that while IgA antibodies produced against BSA were elevated, IgM antibodies to BSA were not (Karjalainen et al. 1992a; Levy-Marchal et al. 1995a; Saukkonen et al. 1995; Saukkonen et al. 1998a). It has been observed that bovine milk protein hydrolysates are competitively inhibiting ABBOS binding to antibody. Therefore, to warrant that hydrolysed infant formulas are free of cross-reactive ABBOS antibody binding sites, a significant variation in residual reactive sites among hydrolysed BMP requires specific identification (van Beresteijn and Meijer 1996).
While elevation in anti-BLG IgG antibodies was widely demonstrated (Savilahti et al. 1988; Savilahti et al. 1993; Saukkonen et al. 1995; Ahmed et al. 1997; Saukkonen et al. 1998a), IgA antibodies to BLG were considered independently associated with the risk of developing the disease (Dahlquist et al. 1992; Saukkonen et al. 1995). Similarly, IgA antibodies to whole BMP in recently diagnosed young diabetic children were high (Savilahti et al. 1988; Dahlquist et al. 1992; Savilahti et al. 1993; Saukkonen et al. 1998a) and were also considered as an independent disease risk determinant. A few studies were not able to establish associations for anti-BSA antibodies in recently diagnosed children (Ivarsson et al. 1995; Ahmed et al. 1997).

Antibodies to BSA in HLA-DR3 recently diagnosed children were higher than that in both HLA-DR3 non-diabetic children and non-HLA-DR3 diabetics (Krokowski et al. 1995). Identical diabetic sibling pairs for HLA-DQB1* (0201, 0302, 0602/03) alleles associated with the risk or protection from diabetes have shown elevated antibodies to BMP than their HLA-DQB1 matched sibling control group which was an independent risk factor regardless of feeding practices (Kostraba et al. 1993; Saukkonen et al. 1998b). Nevertheless, a French study was not able to establish a significant association between antibodies to BSA and HLA-DQB genotype (Levy-Marchal et al. 1995a).
1.5.3.2 Cellular immunity

Cellular immune mechanisms are implicated as the main mediator of the pancreatic β-cells autoimmunity process. A dichotomous relation has been established between autoantibodies circulating to glutamic acid decarboxylase and peripheral-blood T-cell proliferation in at-risk relatives of persons with diabetes (Harrison et al. 1993). Mean stimulation index of proliferation of T-cell to β-casein, but not to BSA or BMPS is significantly higher in newly diagnosed children with diabetes than in non-diabetic children or patients with autoimmune thyroid disease (Cavallo et al. 1996).

Cellular responsiveness of peripheral-blood mononuclear cells was not observed to BSA or common food antigens. Enhanced cellular response with BLG in newly diagnosed diabetic children was significant and was not associated with HLA-DQB1* TIAD risk allele (Vaarala et al. 1996). No cellular response was observed in HLA and/or age matched at-high risk controls, but peripheral lymphocytes T-cell produced from newly diagnosed diabetic children were significantly responsive to insulin B chain 9-23 amino acid residue (Alleva et al. 2000).

A few studies have not been able to demonstrate either cellular response (Atkinson et al. 1993) or humoral response to BSA or ABBOS peptide (Atkinson et al. 1993; Ivarsson et al. 1995). Elevated cellular and humoral immune responses to BSA among patients with other autoimmune diseases (chronic autoimmune thyroiditis, rheumatoid arthritis, and systemic lupus erythematosus) and at the risk of developing diabetes may defer its possible role as a diabetogenic factor (Atkinson et al. 1993).
One study has indicated that cellular immunity and humoral immunity to a particular food antigen is not limited to diabetic persons. Cellular responsiveness to α and β-casein found higher in both diabetic and non-diabetic children had HLA-DR3 alleles, with no difference in the cellular response in either groups (Sarugeri et al. 1999). Lack of relationship between antibodies to BSA and ICA may indicate that humoral immunity to BSA may be a sign of an unspecific and a broad defect in the course of immunologic tolerance (Atkinson et al. 1993; Luhder et al. 1994; Ivarsson et al. 1995).

1.5.3.3 Secretory immune system

Secretory IgA is one dominant characteristic humoral factor of the local immune system in the body secretions. Saliva, for instance may act as a first-line of defence against local infections as well as prevent access of foreign antigenic factors to the immune system (Roitt et al. 2001). The levels of sIgA, following the development and maturation of the salivary glands, has a parabolic rapport with age (Smith et al. 1987; Ben-Aryeh et al. 1990; Kugler et al. 1992; Percival et al. 1997). Thus, sIgA has been widely used as a biomarker in biobehavioural studies (Ben-Aryeh et al. 1984; Worthman et al. 1990; Hertsgaard et al. 1992; Shirtcliff et al. 2000; Shirtcliff et al. 2001), determination of toxins and pollutants (Gilfrich et al. 1981; Bauer et al. 1983), and as a diagnostic tool (Behets et al. 1991; Ciclitira and Ellis 1991; Kozlowski and Jackson 1992).
The synthesis of sIgA is directly associated with foreign factors, given a high sIgA synthesis rate and a short half-life, and associated directly to the activation of T and B cell activation as well (Smith et al. 1987; Paul 1993). Although, antibody immune responses at various mucosal effector sites are antigen type and dosage dependent, the whole mucosal immune system faraway from the sensitisation site can be engaged by the sensitisation of a specific site of the system that may well present a thorough view of the performance of the entire mucosal immune system (Externest et al. 2000). A salivary-based ELISA has been used in the determination of antibodies produced against gliadin in patients with celiac autoimmune disease (Ciclitira and Ellis 1991; Fasano et al. 2003). Given that salivary samples collection is a non-invasive, stress-free, simple method, and can be easily collected and stored (James-Ellison et al. 1997) it may, therefore constitute an alternative system to detect humoral immunity in diabetic children.

### 1.5.4 Bovine insulin: The contender counterpart

There is a growing evidence indicating that insulin and its precursor proinsulin particularly the 9-23 immunodominant residue peptide of the B chain are widely anticipated as potential autoantigens capable of initiating the destruction of insulin secreting pancreatic beta cells sanctioning other pancreatic proteins to become the primary target leading to the disease (Wegmann et al. 1994; Abiru et al. 2000; Alleva et al. 2000; Wong et al. 2002; Kent et al. 2005; Nakayama et al. 2005). It has been also anticipated that insulin becomes a target ensuing the initiation of the autoimmune process by another autoantigen (Atkinson et al. 1986; Srikanta et al. 1986; Vaarala et al. 1998; Komulainen et al. 1999; Alleva et al. 2000; Kimpimaki et al. 2002; Juvenile Diabetes Research Foundation International 2005b).
It has been recently demonstrated that breeding a knockout of the insulin 1 gene and a knockout of the insulin 2 gene into NOD mice has prevented most progression to diabetes in the latter and has accelerated the development of the disease and the development of the insulin autoantibodies in the former (Nakayama et al. 2004; Nakayama et al. 2005), however insulin knockouts were not able to prevent all pancreatic autoimmunity, but were able to prevent islet-specific autoimmunity.

Neither was deletion of the GAD65 gene able to affect the disease occurrence in NOD mice indicating that GAD65 expression is not a primary autoantigen for the development of the disease (Kash et al. 1999). These findings indicate that insulin and proinsulin molecules have a target sequence of the pancreatic autoimmune process that bolsters the hypothesis that, if primary autoantigens do exist for specific autoimmune process, proinsulin is a primary autoantigen of the NOD mouse. This constitutes a novel ground for possible disease prevention by the application of deletion therapy (Nakayama et al. 2005).

The intensity of autoimmune T-cell mediated response triggered by a certain autoantigen is proportional to the level of that antigen expressed in the target tissue. It has been suggested that islet-specific T-cell mediated immune response candidate autoantigens in persons with autoimmune diabetes are important in the destruction of the pancreatic beta-cells (Van Vliet et al. 1989; Roep et al. 1990; Neophytou et al. 1996; Roep et al. 1999).
In humans, the expression of insulin is adequate to target a tissue to T-cell mediated pathology. Cellular immunity to proinsulin, peptide (amino acids 24-36) was virtually limited entirely to individuals at risk of developing the disease. Newly diagnosed children with diabetes developed a substantial cellular immune response to 9-23 immunodominant amino acid region of the insulin B chain (Alleva et al. 2000). Recent findings support the hypothesis that insulin may be a primary autoantigen. It has been demonstrated that a high level of T-cell clonal expansion was observed in pancreatic lymph nodes from diabetic patients with HLA-DR allele that was not observed in controls. Clonally expanded CD4\(^+\) clones recognised specifically the insulin A chain 1-15 amino acid residue.

A prospective cohort study has demonstrated that cellular and humoral immune response to bovine insulin at three months of age was higher in infants exposed to bovine milk-based formula than that in infants exclusively breastfed. At three months of age, infants exposed to bovine milk-based formula showed higher anti-bovine insulin IgG antibodies levels than in infants who received casein hydrolysate-based formula, but there was no difference in cellular response among both groups (Paronen et al. 2000b). Early exposure of infants to bovine milk proteins resulted in decreased levels of antibodies produced to bovine insulin at 18 months of age. Although dietary antigens present in human breast milk may have a role in early tolerance induction resulting in immunisation, that role remains intriguing and requires further investigation (Paronen et al. 2000a).
A human workshop on T cell has pointed out that populations diversity and inability to differentiate between non-diabetic controls and newly diagnosed patients, and difficulties in identifying and preparing candidate autoantigens; as well as interlaboratory variation and availability of suitable assays for standardised use may explain conflicting findings and problems associate with autoreactive T cell assays in T1ADM (Roep et al. 1999). It remains difficult to identify which antigens serve as targets for beta-cell destruction in humans with autoimmune diabetes and whether they are the same as those involved in NOD mice (Wegmann and Eisenbarth 2000).

Insulin is a 5,808 Daltons (Harfenist and Craig 1952; Sanger 1958), 51 amino acids, dual-chain hormone; chain A (21 amino acids) and B chain (30 amino acids). Insulin is the main product of the pancreas islet β-cells and is not produced in other tissues in hormonally significant quantities (Wegmann and Eisenbarth 2000). Also, it is necessary for the intestinal growth and maturation (Shamir et al. 2001) as well as for the control of several cellular metabolic courses (Buts et al. 1997a).

Human breast milk contains a stable (5 ng/ml) concentration of immunoreactive insulin (Harrison and Honeyman 1999), whereas bovine milk contains (327 ng/ml) in the first milking. Variation in bovine milk insulin is considerable, particularly during the first period of lactation; bovine milk insulin concentration fell to approximately 50% within the first day postpartum and it reaches a stable concentration on day seven postpartum (46 ng/ml; about 14% of its first milking concentration). Bovine milk insulin concentration is almost 100 times higher than that of serum insulin, which suggests a specific mechanism of transferring insulin from serum to milk (Aranda et al. 1991). Immunisation to insulin arises primarily from exposure to bovine insulin and that may play a key role in the pancreatic autoimmune process (Vaarala et al. 1999; Paronen et al. 2000b).
Although human, bovine and porcine insulin share high degree of sequence homology, they differ at certain amino acid residues. Human insulin differs only at one B30 (Threonine) amino acid residue from that of porcine insulin (Alanine), whereas human insulin differs at three residues from that of bovine insulin (A8 [Threonine replaced with Alanine], A10 [Isoleucine replaced with Valine] and B30 [Threonine replaced with Alanine], respectively). It has been demonstrated that two-fold increase in the exposed area of the bovine insulin compared to that of the porcine insulin analog was accompanied with a significant reduction of the hydrophilic area on the same insulin hexamer surfaces, given changes in amino acid residues (Yip et al. 1998).

The absence of prominent differences between human and porcine insulin of similar purity in terms of their antigenicity (Larkins et al. 1986) is likely due to the closer structure of porcine insulin to that of human insulin (Little et al. 1977). The discernible variation between bovine insulin, human insulin and porcine insulin indicates that the physical characteristics of insulin rather than the variation in the amino acid sequence may determine its immunogenicity (Kurtz et al. 1985). A comprehensive literature review available up to 2002 aiming at assessing the effect of different insulin species has indicated that antibodies produced against insulin did not show relevant dissimilarities between most of the investigated purified porcine insulin and semisynthetic human insulin although the methodological quality of most studies was poor (Richter and Neises 2004). Nonetheless, it has been demonstrated that conventional bovine insulin contains immunogenic amounts of proinsulin to humans when administered to individuals with T1ADM and is more immunogenic than purified pork insulin (Kawazu et al. 1979; Kurtz et al. 1980).
Bovine insulin is a small protein of 5,808 Daltons (Harfenist and Craig 1952; Sanger 1958) and cannot elicit an antibodies immune response by itself; this requires coupling to a carrier protein such as BSA (Harlow and Lane 1988) of 66430.3 Daltons (Carter and Ho 1994). Modification of both BSA (Teale and Benjamin 1976) and bovine insulin (Speth and Lee 1984) with various binding molecular as well as heat processing have a substantial effect on their immunogenic response (Hanson and Mansson 1961; Karjalainen et al. 1992a; Alting et al. 1997). These modifications either alter regions of the immunogen to present better sites for T-cell binding or expose new epitopes for B-cell binding (Harlow and Lane 1988).

The application of the suitable heating procedure may play a role in determining the diabetogenicity degree of certain dietary constituents (Strand 1994). Studies on the thermo lability of insulin and its role in the diabetogenicity of insulin remain scarce. Nonetheless, its widely accepted that for insulin to maintain its viability, insulin-dependent diabetic patients are encouraged not to store insulin vials under extreme temperatures (<2 or > 30 ºC) (American Diabetes Association 2002).

Furthermore, insulin suspensions exposed to temperatures more than 25 ºC for extended periods may become difficult to homogenise, whereas exposing insulin to 50 ºC or more leads to the coagulation of the insulin suspensions (Brange 1987). Studies on the thermo lability of insulin and its role in altering the immunogenicity of the insulin molecule remain scarce. It is, therefore, pivotal to examine the effect of both modification and thermal processing on the diabetogenicity of insulin, given that canned infant milk formulae undergo a wide range of both large and home scale heat processing (Pisecky 1997).
1.6 Proteins modification: Enhanced immunogenicity

Albumin is the most copious protein of the blood circulatory system. Bovine serum albumin constitutes only one per cent of whole bovine milk (Carter and Ho 1994; National Health and Medical Research Council 2003). The predominantly alpha-helical polypeptide globin structure of BSA (Carter et al. 1989) consists of 607 amino acids (66430.3 Daltons) and seventeen disulphide bridges which form nine loops creating three domains (Feldhoff and Peters 1975; McLachlan and Walker 1977; Carter et al. 1989; Hirayama et al. 1990; Carter and Ho 1994). Bovine serum albumin amino acid sequence of peptide (1-24) has a high degree of similarity with that of human serum albumin. They only differ at four amino acid residues (2 [Threonine-Alanine], 6 [Isoleucine-Valine], 18 [Histidine-Asparagine] and 21 [Glycine-Alanine] respectively) (Bradshaw and Peters 1969).

Native BSA binds heterogeneously with detergents involving many sites of varying affinity; given native BSA has four distinct binding sites for detergents. The acceptor activity of BSA with detergents occurs without conformational changes and results in major denaturation (Nozaki et al. 1974). The modification of arginine at its amine terminal first enhances the acceptor activity of BSA significantly (Leibowitz and Soffer 1971). Glycation reaction with BSA involves reversible and irreversible formation of modified BSA which occurs mainly on arginine residues (Lo et al. 1994). The increased levels of advanced glycation end products are implicated in diabetes vascular complication (Thornalley et al. 2003).
Restricted antibodies population to isolated different antigenically active BSA fragments representing BSA on both domains and subdomains showed that some regions of the molecule refold more rapidly than other domains. However, the entire polypeptide chain was not necessary for the reformation of native BSA structure indicating a possible interdomain influence. It has been demonstrated that when the antigenic sites of modified BSA with methoxypolyethylene glycol were destroyed, modified BSA did not induce any immune responses compared to a strong native albumin immune response (Teale and Benjamin 1976).

Specific protein phosphatases may have a certain binding site or sites for porcine insulin and that modified insulin with phosphatases may enhance catalytic activity. Porcine insulin was capable, in vitro, of activating and binding to purified rabbit skeletal muscle phosphatase. It was temperature, time, and concentration dependent and was not mimicked with either insulin A chain or insulin B chain or BSA. The activation phenomena may be prevented by insulin antisera (Speth and Lee 1984). The kinetics of tryptic hydrolysis of the arginyl and lysyl bonds at bovine insulin B chain (B22, and B29) have demonstrated that the zinc-free bovine insulin B chain folded into the secondary and tertiary characteristic of the bovine insulin. The basic residues of insulin become more unavailable to tryptic hydrolysis than the matching residues in the oxidised chain (Wang and Carpenter 1969). Substantial interactions with other ingredients in food systems may take place, resulting in modified behaviour of the proteins (Kinsella and Whitehead 1989) and therefore may play a crucial role in our understanding toward the disease aetiopathogenesis.
1.7 Thermal processing: Altered immunogenicity

Human breast milk is the perfect initial food to meet the rapid growth infants experience during their first year. If certain health or personal conditions in either mother or infant prevent breastfeeding, bovine milk-based infant formula is the formula of choice (Wold and Adlerberth 2000; Oddy 2002). Mature human milk differs considerably in composition compared to that of other species. It particularly contains protein and sodium chloride at very low concentrations and high concentrations of lactose and oligosaccharides (National Health and Medical Research Council 2003).

In spite of all efforts to duplicate human milk, it nevertheless remains challenging. While emphasising “closeness to breast milk”, a report has warned against continuous manufacturers’ violation of the international code on the marketing of human breast milk substitutes (Mayor 2004). Requirements to assimilate human milk high lactose content have made infants formulas as one of the most difficult-to-dry foods. Modern infant formulas require both large scale and home food processing that can bring about extreme alteration in the chemical composition of food products (Rechcigl 1982).

Bovine milk is a complex polydispersion system. Individual components of milk have various impacts on its physical properties. Therefore, the variation in the composition of the milk system has a significant impact on its properties and thus on the properties of the final product (Pisecky 1997). Bovine milk contains more than 25 separate proteins, 80% of them are heterogeneous casein occurring as a micellar complex and whey proteins form the remaining (20%) of the milk protein components (Whitney 1988). Heat processing is the most commonly used in canned bovine milk-based foods processing technologies and is attributing to some undesirable possibly severe quality defects in bovine milk, a heat sensitive liquid, including the denaturation of its proteins (Pisecky 1997).
The effect of heat depends among other factors on pH (Law and Leaver 2000), heating temperature, presence or absence of co-denaturation associate, and industrial thermal pre-treatment (Bertrand-Harb et al. 2002). Therefore, it is critical to identify possible conformational changes of milk proteins resulting from heat processes (Relkin 1996).

Bovine milk proteins are implicated in causing allergenic reactions in infants (Goldman et al. 1963; Kletter et al. 1971b; May et al. 1982; Bahna and Gandhi 1983) as well as in inducing a specific immune response in humans (Bahna and Gandhi 1983; Karjalainen et al. 1992b; Saukkonen et al. 1994; Levy-Marchal et al. 1995a). The immunogenicity of antigens can be often substantially altered by slight changes in the structure of antigen; many molecules can be made more immunogenic by heat denaturation, given that protein antigens aggregate caused by heat processing are usually more immunogenic.

This treatment can change the structure of many compounds, particularly proteins, and expose new epitopes. Even small epitope structural changes can prevent antigen recognition and can prevent antibodies differentiating between conformations of protein antigens. These modifications either alter regions of the immunogen to present better sites for T-cell binding or expose new epitopes for β-cell binding (Harlow and Lane 1988; Harlow and Lane 1999).

Immune electrophoretic studies on bovine colostrum and other milk products showed that BSA (a highly folded tertiary conformation of 608 amino acid hydrophilic whey protein that renders protection to casein) (Feldhoff and Peters 1975; Hirayama et al. 1990; Pisecky 1997) is destroyed at 70 to 80°C for fifteen minutes. Beta-casein, a 209 amino acids molecule exists in an open structure, and beta-lactoglobulin are very stable. They retain their total solubility and antigenicity even under extreme heat treatment for extended periods of time (120°C/15 min and 100°C/15min, respectively) (Hanson and Mansson 1961).
Radial immunodiffusion assay for BSA has found that bovine-based infant formulae, ultra-high temperature (UHT) milk, ultrapasteurised milk, and milk boiled at elevation (<6400 feet) were free of BSA. Heat processing at various temperatures and exposure time (84, 85 and 86°C/>4 min, 89°C/>3 min, 90 °C/90 Seconds and 94°C/60 Seconds) and have rendered milk free of BSA (Strand 1994). Thermal processing of canned infant formulae as well as liquid formulae at different temperatures and exposure time has rendered milk free of BSA (Monte et al. 1994; Strand 1994).

The physical nature of the antigen affects its immunogenicity (Saukkonen et al. 1994). Serum antibody titre levels produced against pasteurised bovine milk were the highest compared to antibodies produced against heat processed bovine milk proteins (May et al. 1982). Antibodies to BSA are common in the general population, given the detection of antibodies associated with diabetes is technically demanding (Levy-Marchal et al. 1995a). Although bovine serum albumin has many epitopes capable of inducing a broad range of high and low affinity general antibodies population not only to conformational epitopes, but also to denatured BSA it can be also recognised by the general antibodies population (Karjalainen et al. 1992b; Savilahti et al. 1993; Saukkonen et al. 1994).

Heat processed milk contains active (Saperstein and Anderson 1962), but less antigenic BLG and alpha-lactalbumin (ALA) in both liquid and powder form and results in less frequent reactions than pasteurised milk (Crawford 1960). Thermal processing of enzymatic hydrolysates of the whey protein at 90°C/10 minutes has reduced its antigenicity significantly (Boza et al. 1994). Individuals sensitive to BSA may tolerate heat processed bovine milk proteins; tolerance was reduced for individuals responsive to other protein fractions (Crawford 1960). Animal studies (Kilshaw et al. 1982; Heppell et al. 1984) have shown that unlike pasteurised whey proteins or skimmed milk, antibodies produced to extremely heat treated whey proteins (100 or 115°C for 30 minutes) were very low or undetected. If detected antibodies were residual casein specific that indicates an extensive denaturation of these proteins.
1.8 Prevention trials: Pros and cons

Most of the evidence on the diabetogenicity of the dietary proteins particularly bovine milk proteins has come from retrospective studies, yet these findings remain widely discrepant and controversial. Discrepancy in these findings in both human and animal retrospective studies on autoimmune diabetes indicates variations in the diabetogenicity of milk proteins. In addition, this discrepancy is a result of a number of factors. They include: (1) the actual exposure to diabetogenic factors in certain populations but not in others (Norris et al. 2003), (2) timing of exposure to foreign environmental factors (Verge et al. 1994; Kimpimaki et al. 2001) (3) study design, (4) analysis methods (Karjalainen et al. 1992b), and (4) differences between populations examined (Esfarjani et al. 2001).

The secondary prevention trials of diabetes are models to obtain large cohorts for reliable evaluation of the secondary prevention. The European Nicotinamide Diabetes Intervention Trial (ENDIT), a multicentre five-years randomised double-blind placebo-controlled intervention trial of nicotinamide, has concluded that nicotinamide was ineffective at preventing or delaying the clinical onset of T1ADM in individuals with a first-degree family history of the disease (Gale et al. 2004).

Similarly, the American Diabetes Prevention Trial-type 1 (DPT-1), a double-blind, placebo-controlled clinical trial, has concluded that the administration of oral insulin was ineffective in delaying or preventing T1ADM in first and second-degree non-diabetic relatives at risk for diabetes (Skyler et al. 2005). Findings of secondary prevention trials on the role of administration of either oral or intranasal insulin may have on the destruction of the pancreatic beta cells were contradictory.
The Nasal Insulin in Prevention of Type 1 Diabetes Trial, a randomised controlled clinical trial under the umbrella of the Finnish Diabetes Prediction and Prevention (DIPP), has indicated that a decreased response of the first-phase insulin can be an initial phenomenon in the course of prediabetes in young children (Kupila et al. 2001; Keskinen et al. 2002; Juvenile Diabetes Research Foundation International 2005a) and the Melbourne Nasal Insulin, a crossover randomised controlled clinical trial, has suggested that intranasal insulin does not accelerate the loss of beta-cell function in individuals at risk for T1ADM and induces immune changes consistent with mucosal tolerance to insulin (Harrison et al. 2004). Both the Italian IMDIAB study group (Pozzilli et al. 2000) and the French Diabetes Insulin Oral Group (Chaillous et al. 2000), multicentre randomised controlled clinical trials, have demonstrated that the administration of oral insulin initiated at the clinical onset of T1ADM is not able to prevent the deterioration of the pancreatic beta-cells.

Reviews of the designs and inclusion criteria of the secondary prevention trials of diabetes have indicated that there were pros and cons to the initiation of each of these studies (Knip and Akerblom 1998; Rosenbloom et al. 2000; Schatz 2002). Therefore, there are lessons to be learned as negative outcome in these studies is reported (Pozzilli 2002). The establishment of a number of coordinated secondary prevention intervention trials remain an appropriate response to this life-long overwhelming disease (Gale et al. 2004; Harrison et al. 2004).
Primary prevention trials aiming at warding off the disease from occurring in high-risk populations prior to any detectable indications of prediabetes islet β-cell autoimmunity take place constitutes a main challenge to the ongoing research efforts. Two human primary prevention trials have been established: the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) and the Type 1 Diabetes TrialNet. The former is the first dietary intervention primary prevention study warranted to prove or disprove whether avoidance of bovine milk-based formula has an effect on the development of T1ADM in children.

This trial has shown that substituting BMP with hydrolysed casein-based infants formula over the first six to eight months of age might protect genetically susceptible children from developing pancreatic β-cell autoimmunity (Akerblom et al. 1999; Akerblom et al. 2005). The latter is a multicentre study group established recently to conduct natural history, preventive research and study intervention therapies for people with newly diagnosed diabetes (Type 1 Diabetes TrialNet n.d.).

The Triggers and Environmental Determinants in Diabetes of the Young (TEDDY), a consortium of six international centres, has been established to examine, confirm and extend the findings of the ongoing and the future prospective cohorts. This consortium is to coordinate the ongoing international efforts to identify infectious agents, dietary factors, or other environmental factors that contribute to the risk of T1ADM in individuals with genetic susceptibility (National Institute of Health 2003).
Enhancing the understanding of the biological history of the preclinical stage of T1ADM will achieve ample insights into disease immunopathogenesis. The identification of individuals for prevention trials may lead directly to more efficient early diagnosis prior to the manifestation of the disease. Nevertheless, the ability to predict the disease without a capability of primary prevention raises a number of ethical issues including induced stress, changes in the lifestyle and insurability (Schatz et al. 2000). Lack of effective intervention remains the single most significant barrier against population wide scale trials (Schatz et al. 2002).

Given that large-scale trials screening stages and prevention procedures are based on the assumption of eventually positive findings, the possibility to fail to prevent the disease constitute a threat, anxiety, uncertainty, conflict and depression to individuals at high risk of developing the disease and to their families. Therefore, families participating in these trials require psychological consultation and support at every stage of the study (Weber and Roth 1997; Roth 2001). Further investigations into the underlying mechanisms of the disease are essential prior to undertaking additional intervention trials (Atkinson and Gale 2003).

Overcoming the inherited ethical issues accompanying the likelihood of instigating the awareness of risk in healthy persons and possible false positivity requires minimising controversy surrounding the prerequisites of any disease prevention trials (Rosenbloom et al. 2000). Integration of behavioural research (Johnson 2001) as well as more fundamental research are justified prior to effective and safe prevention trials commence (Dahlquist 1999). Curtailing the duration of time-consuming clinical trials is essential to set up surrogate markers of clinical T1ADM and is one approach that will ease the initial assessment of potential intervention modalities and selection of the most promising therapies for full-scale testing with overt disease as the endpoint (Knip and Akerblom 1998).
1.9 To sum up

Human studies on T1ADM (Kimpimaki et al. 2001; Norris et al. 2003) and human studies on celiac disease (Ivarsson et al. 2002) as well as animal studies (Elliot and Martin 1984; Elliot et al. 1988; Johnston and Monte 2000; Scott et al. 2002) have concluded that the risk of developing the disease was reduced if the diabetogenic dietary agent, such as BMPs and cereals was introduced while the newborn was still breastfed independent of the age of exposure. These findings indicate a possible diabetogenicity of certain early infant foods, but it should not be misinterpreted by parents and the public (Atkinson and Gale 2003).

Compliance with the current six months infant exclusive breastfeeding recommendations of the World Health Organisation should be maintained (World Health Organization 2002). The need for complementary nourishments afterwards in combination with continuous breastfeeding up to two or three years of age or beyond should also be recognised (National Health and Medical Research Council 2003; World Health Organization and United Nations Children's Fund 2003). Success in the ongoing research on preventing the occurrence of the disease in animals and the exciting findings about its causes are imperative for diagnosis, treatment, and minimising or even preventing the onset of the disease in humans.
2 Type 1 autoimmune diabetes: Implications of worldwide imported milk and milk protein consumption

2.1 Abstract

Objective: Claims of adverse health effects from milk ingredients are on the increase. The increasing incidence of type 1 diabetes mellitus due to pancreatic autoimmunity has been related to the source and amount of milk proteins consumption (MPC). This study examined the possible association between imported milk excluding butter (IMEB) and MPC and the occurrence of the disease.

Design and methods: Data for type 1 diabetes mean incidence and percent increase in the disease incidence per year in children aged 14 years or less were taken from the Onkamo study (1999). Data for IMEB and MPC were obtained for each individual country from FAO food balance sheets. An average percent relative change in IM and MPC for each country was calculated for the period matching that of the Onkamo study. Correlations between both mean and relative increase in the disease incidence, and both mean and average percent relative change in IMEB; and mean and average percent relative change in MPC were computed. A linear regression model was used to address the strength of that relationship.

Results: The data fit a linear regression model. Correlation between disease mean incidence and both relative change in IMEB and mean MPC was $r = 0.648$ ($P = 0.05$), and $r = 0.595$ ($p = 0.05$), respectively. A significant inverse association ($r = -0.657$, $P = 0.05$) was established between the increase in diabetes incidence and mean MPC.
**Conclusion:** Worldwide changes in both imported milk proteins and milk protein consumption may have a possible link to mean disease incidence as well as the global increase in type 1 diabetes mellitus.
2.2 Introduction

Type 1 autoimmune diabetes affects approximately 0.5-1% of the general population throughout its lifetime, and is marked by obvious geographic, ethnic and racial variation (Rewers and Klingensmith 1997; Onkamo et al. 1999; Karvonen et al. 2000). This indicates an important interplay between genetic susceptibility and environmental factors in the initiation and development of the disease.

All Nordic countries (Norway, Denmark, Sweden, and Finland) have a similar genetic background and relatively similar breastfeeding practices (International Dairy Federation 1993; Freysteinsson and Sigurdsson 1996; Elliot et al. 1999; Thorsdottir et al. 2000; Erkkola et al. 2005), nevertheless the occurrence of type 1 autoimmune diabetes (T1ADM) varies considerably in these countries. Whereas the relative increase in the disease occurrence is relatively similar, the occurrence rate of the disease in Iceland is 50% lower than that in other Nordic countries (Onkamo et al. 1999).

The hypothesis of an inverse correlation between the protective effect breastfeeding may have on the development of T1ADM and the implication of the early introduction of bovine milk proteins at early age with the disease aetiopathogenesis has undergone worldwide extensive investigation (Borch-Johnsen et al. 1984; Gerstein 1994; Verge et al. 1994; Norris and Scott 1995; Gimeno and de Souza 1997; Meloni et al. 1997; Couper et al. 1999; Hummel et al. 2000; Thorsdottir et al. 2000; Akerblom et al. 2005).
Animal and human studies were able to establish a significant association between differences in the amount of bovine milk protein consumed, which may govern the intestinal transmission of macromolecules, and the occurrence of the disease (Westrom et al. 1985; Scott 1990; Dahl-Jorgensen et al. 1991). Studies in Iceland were able to demonstrate that only beta-casein variants may be correlated with lower disease occurrence in Iceland (Elliot et al. 1999; Thorsdottir et al. 2000; Birgisdottir et al. 2002).

If bovine milk proteins are diabetogenic, the controversy and discrepancy of the findings of these studies may indicate variations in diabetogenicity of bovine milk proteins. The purpose of the present study was to investigate, retrospectively, possible associations for worldwide mean and relative change in both imported milk excluding butter (IMEB) and milk protein consumption (MPC) per capita per day, and both mean and global increase in the disease occurrence.

2.3 Research design and Methods

Data for the T1ADM mean occurrence, and percent increase in disease occurrence per year in children aged 14 years or less were taken from the Onkamo study (Onkamo et al. 1999), which has a strict inclusion criteria. Data for IMEB (trillion metric tonnes), and MPC gram (per capita per day) were obtained from FAO food balance sheets (Food and Agriculture Organisation of the United Nations 2004).
FAO food balance sheets do not provide specific data on a specific region or an ethnic group in a specific country. Therefore, we assumed that available FAO balance sheets data on mean and percent change in both IMEB and MPC for West Australia, Lima Peruvians, and Yemenite Israelites represent that of Australian, Peruvian, and Israeli populations. Countries with more than one region included in the Onkamo study were excluded from the present study.

Twelve countries were included to represent the global variation in both the mean and the increase in disease occurrence. Mean of both IMEB excluding butter and MPC per capita per day for each individual country as well as average percent relative change in IMEB and MPC for each country were calculated for the period matching that of the Onkamo study, given that the reference year was the study starting year of each study included in the Onkamo study.

2.3.1 Statistical analysis

Assuming that errors were normally distributed and that data fit a linear regression model, correlations between both mean and relative increase in disease occurrence; and both mean and average percent relative change in IMEB as well as mean and average percent relative change in milk protein consumption MPC were computed by the bivariate correlations procedure. The One-Sample T-Test was applied to determine significant differences between means by using the Statistical Package for Social Sciences (SPSS). Data were considered statistically significant at P<0.05.
2.4 Results

There was a significant and strong positive correlation between mean disease occurrence; and both relative change in IMEB and mean MPC (r = 0.648, P = 0.05, and r = 0.595, p = 0.05 respectively). Although not significant, there was an inverse association between mean disease occurrence and both mean IMEB and the relative change in MPC (r = -0.214, P = 0.05, and r = -0.313, P = 0.05, respectively).

For relative increase in occurrence of T1ADM in children aged 14 years or less, there was a strong significant inverse correlation with mean MPC (r = -0.657, P = 0.050). An inverse and not significant correlation was established with relative change in IMEB excluding butter (r = -0.226, P = 0.050) and a positive not significant correlation with relative change in MPC (r = 0.279, P = 0.050).

Mean and relative increase in the disease occurrence is shown in Table 2-1. It shows that while Iceland and Finland have the same relative increase in the disease occurrence, Finland has the highest disease occurrence. Imports of milk excluding butter into France was the highest among all countries (2.63 trillion ton, 95% CI 2.16; 3.11, P = 0.000) (Table 2-2) with relatively low variations in IMEB (0.03%) (Table 2-3), whereas imports of milk into Iceland was the lowest (0.001 trillion metric tonnes, 95% CI -2.4; -1.9, P =0.000) and relative change in IMEB was the lowest (0.02%, 95% CI -0.17; 0.22, P 0.809).
Although imported milk excluding butter into Finland was relatively low (0.047 trillion metric tonnes, 95% CI 0.35; 0.53, P = 0.000), relative change in IMEB in Finland was higher than any other country (6.26%, 95% CI 5.05; 7.42, P = 0.0000). This was higher than that of worldwide relative change in IMEB by more than 5-fold. Relative change in IMEB in Hungary was 0.044 trillion metric tonnes (-0.60; 95% CI -0.81; -0.46, P = 0.0000).
Table 2-1 Worldwide mean (per 100,000) and relative increase in occurrence of type 1 ‘autoimmune’ diabetes among children aged 14 years or less modified from the Onkamo study (1999). Relative increase in the disease occurrence in Peru (Lima), Australia (West), and Israel (Yemenite Jews) is assumed to represent that of the whole populations of these countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Study period</th>
<th>Mean disease occurrence</th>
<th>% Increase in disease occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>1978-87</td>
<td>6.1</td>
<td>8.5</td>
</tr>
<tr>
<td>Peru: Lima</td>
<td>1985-94</td>
<td>0.5</td>
<td>7.7</td>
</tr>
<tr>
<td>Australia: West</td>
<td>1985-92</td>
<td>14.9</td>
<td>6.3</td>
</tr>
<tr>
<td>Libya</td>
<td>1981-90</td>
<td>8.7</td>
<td>6.3</td>
</tr>
<tr>
<td>France</td>
<td>1988-95</td>
<td>8</td>
<td>3.9</td>
</tr>
<tr>
<td>Israel: Yemenite</td>
<td>1965-93</td>
<td>5</td>
<td>3.2</td>
</tr>
<tr>
<td>Norway</td>
<td>1973-82</td>
<td>20.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Austria</td>
<td>1979-93</td>
<td>7.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Finland</td>
<td>1965-96</td>
<td>30.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Iceland</td>
<td>1970-88</td>
<td>9</td>
<td>2.3</td>
</tr>
<tr>
<td>Sweden</td>
<td>1978-92</td>
<td>24.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Malta</td>
<td>1980-96</td>
<td>14.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Global</td>
<td></td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>
Table 2-2 Worldwide mean imported milk excluding butter (IMEB) (Trillion metric tonnes). Mean IMEB for each individual country modified from Food and Agriculture Organisation of the United Nations (2004) and calculated for the period matching that of the Onkamo study (1999). The populations arranged in descending order in accordance with the relative increase in the disease occurrence per year. Relative increase in the disease occurrence in Peru (Lima), Australia (West), and Israel (Yemenite Jews) assumed to represent that of the whole populations of these countries. All data were statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Country</th>
<th>Mean imported milk excluding butter (trillion metric tonnes)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>0.044</td>
<td>0.002</td>
</tr>
<tr>
<td>Peru</td>
<td>0.314</td>
<td>0.000</td>
</tr>
<tr>
<td>Australia</td>
<td>0.214</td>
<td>0.000</td>
</tr>
<tr>
<td>Libya</td>
<td>0.298</td>
<td>0.000</td>
</tr>
<tr>
<td>France</td>
<td>2.626</td>
<td>0.000</td>
</tr>
<tr>
<td>Israel</td>
<td>0.095</td>
<td>0.000</td>
</tr>
<tr>
<td>Norway</td>
<td>0.028</td>
<td>0.005</td>
</tr>
<tr>
<td>Austria</td>
<td>0.277</td>
<td>0.000</td>
</tr>
<tr>
<td>Finland</td>
<td>0.047</td>
<td>0.000</td>
</tr>
<tr>
<td>Iceland</td>
<td>0.001</td>
<td>0.000</td>
</tr>
<tr>
<td>Sweden</td>
<td>0.196</td>
<td>0.000</td>
</tr>
<tr>
<td>Malta</td>
<td>0.050</td>
<td>0.000</td>
</tr>
<tr>
<td>Global</td>
<td>0.229</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 2-3 Worldwide mean relative change in mean imported milk excluding butter (IMEB) (Trillion ton) modified from Food and Agriculture Organisation of the United Nations (2004) and calculated for the period matching that of the Onkamo study (1999). The populations arranged in descending order in accordance with the relative increase in the disease occurrence per year. Relative increase in the disease occurrence in Peru (Lima), Australia (West), and Israel (Yemenite Jews) assumed to represent that of whole populations of these countries. Data were considered statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Country</th>
<th>Mean relative change in imported milk excluding butter</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>-0.06</td>
<td>0.000</td>
</tr>
<tr>
<td>Peru</td>
<td>0.03</td>
<td>0.418</td>
</tr>
<tr>
<td>Australia</td>
<td>0.01</td>
<td>0.602</td>
</tr>
<tr>
<td>Libya</td>
<td>0.02</td>
<td>0.050</td>
</tr>
<tr>
<td>France</td>
<td>0.03</td>
<td>0.038</td>
</tr>
<tr>
<td>Israel</td>
<td>-0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>Norway</td>
<td>0.32</td>
<td>0.012</td>
</tr>
<tr>
<td>Austria</td>
<td>0.02</td>
<td>0.144</td>
</tr>
<tr>
<td>Finland</td>
<td>6.26</td>
<td>0.000</td>
</tr>
<tr>
<td>Iceland</td>
<td>0.02</td>
<td>0.809</td>
</tr>
<tr>
<td>Sweden</td>
<td>0.00</td>
<td>0.653</td>
</tr>
<tr>
<td>Malta</td>
<td>0.01</td>
<td>0.010</td>
</tr>
<tr>
<td>Global</td>
<td>1.15</td>
<td>0.000</td>
</tr>
</tbody>
</table>

**Bold** indicates statistical insignificant at P<0.05.
Mean MPC is shown in Table 2-4. The table shows that Icelanders consume more milk proteins (34 g/day/capita, 95% CI 30.9; 33.6, P = 0.000) than that of the Finnish (27 g/day/capita, 95% CI 24.8; 26.0, P = 0.000), whereas the relative change in MPC was declining in both populations (-6.4%, 95% CI -12.5; -8.7 and -8.3%, 95% CI -12.4; -4.9, P = 0.000, respectively) (Table 2-5). In Hungary, MPC was 14.6 g/day/capita (95% CI 6.4; 10.5, P = 0.0000) whereas relative change in MPC was 5-fold higher than that of the global change in MPC (22.8%, 95% CI -4.3; 33.0, P 0.115). This trend was also observed in Israel. Milk protein consumption in Malta and Sweden MPC was among the highest among all studied countries (17.10; 95% CI 16.10; 17.10, 29.36; 95% CI 27.57; 28.75, P = 0.0000, respectively).
Table 2-4 Worldwide mean milk protein consumption (MPC) (g/capita/day). Mean MPC for each individual country modified from Food and Agriculture Organisation of the United Nations (2004) and calculated for the period matching that of the Onkamo study (1999). The populations are arranged in descending order in accordance with the relative increase in the disease occurrence per year. Relative increase in the disease occurrence in Peru (Lima), Australia (West), and Israel (Yemenite Jews) is assumed to represent that of the whole populations of these countries. All data were statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Country</th>
<th>Mean milk protein consumption (g/day/capita)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>14.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Peru</td>
<td>4.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Australia</td>
<td>21.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Libya</td>
<td>10.7</td>
<td>0.000</td>
</tr>
<tr>
<td>France</td>
<td>26</td>
<td>0.000</td>
</tr>
<tr>
<td>Israel</td>
<td>17.4</td>
<td>0.000</td>
</tr>
<tr>
<td>Norway</td>
<td>26.8</td>
<td>0.000</td>
</tr>
<tr>
<td>Austria</td>
<td>21.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Finland</td>
<td>27.7</td>
<td>0.000</td>
</tr>
<tr>
<td>Iceland</td>
<td>34.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Sweden</td>
<td>29.4</td>
<td>0.000</td>
</tr>
<tr>
<td>Malta</td>
<td>17.1</td>
<td>0.000</td>
</tr>
<tr>
<td>Global</td>
<td>22.2</td>
<td>0.000</td>
</tr>
</tbody>
</table>
Table 2-5 Worldwide relative change in mean milk protein consumption (MPC) (g/capita/day). Mean MPC for each individual country modified from Food and Agriculture Organisation of the United Nations (2004) and calculated for the period matching that of the Onkamo study (1999). The populations are arranged in descending order in accordance with the relative increase in the disease occurrence per year. Relative increase in the disease occurrence in Peru (Lima), Australia (West), and Israel (Yemenite Jews) assumed to represent that of the whole populations of these countries. Data were statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Country</th>
<th>Relative change in mean milk protein consumption</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hungary</td>
<td>22.8</td>
<td>0.115</td>
</tr>
<tr>
<td>Peru</td>
<td>-1.9</td>
<td>0.096</td>
</tr>
<tr>
<td>Australia</td>
<td>4.4</td>
<td>0.240</td>
</tr>
<tr>
<td>Libya</td>
<td>-1.1</td>
<td>0.001</td>
</tr>
<tr>
<td>France</td>
<td>0.33</td>
<td>0.020</td>
</tr>
<tr>
<td>Israel</td>
<td>27.4</td>
<td>0.000</td>
</tr>
<tr>
<td>Norway</td>
<td>12</td>
<td>0.011</td>
</tr>
<tr>
<td>Austria</td>
<td>6.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Finland</td>
<td>-8.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Iceland</td>
<td>-6.4</td>
<td>0.000</td>
</tr>
<tr>
<td>Sweden</td>
<td>3.6</td>
<td>0.031</td>
</tr>
<tr>
<td>Malta</td>
<td>-3</td>
<td>0.024</td>
</tr>
<tr>
<td>Global</td>
<td>4.6</td>
<td>0.177</td>
</tr>
</tbody>
</table>

**Bold** indicates statistical insignificant at P<0.05.
Positive trend in MPC with a marked variation among populations is shown in Figure 2-1. Globally, milk protein consumption has increased from 6.7 g/day/cap/day (1961) to 7.2 g/day/cap/day (2001). Milk protein consumption in Finland and Iceland has declined from 31.3 and 37.4 g/capita/day, (1961) to 27.7 and 24.2 g/capita/day (2001) respectively, while consumption has doubled in Libya, and increased by 1.5-fold in Hungary.

Figure 2-1 Worldwide trends (1961-2001) in milk protein consumption (g/capita/day). Data modified from Food and Agriculture Organisation of the United Nations (2004).
2.5 Discussion

All Nordic countries (Norway, Denmark, Sweden, and Finland) have a similar genetic background and relatively similar high breastfeeding practices (International Dairy Federation 1993; Freysteinsson and Sigurdsson 1996; Elliot et al. 1999; Thorsdottir et al. 2000; Erkkola et al. 2005). In Iceland, the occurrence of diabetes is 50% lower than that in other Nordic countries, and approximately one third lower than that in Finland, whereas the relative increase in the disease occurrence among children aged 14 years or less in Iceland and Finland was the same (Onkamo et al. 1999).

Icelanders are amongst the highest in the world in the consumption of bovine milk and the results presented here indicated that although the milk protein consumption in both countries has declined in 2000 compared to that in 1960s, both Icelanders and Finnish populations consume a relatively similar amount of milk proteins. This may explain the similarity in the low relative increase in the disease occurrence in both populations (Onkamo et al. 1999). On the other hand relative change in IMEB in Finland was higher than that of Iceland by a minimum of 300-fold (Table 2-3) which may again explain wide variation in the mean disease occurrence in both populations (International Dairy Federation 1993; Elliot et al. 1999).

It has been demonstrated that the amount of protein available in the intestine of piglets govern the intestinal transmission of macromolecules in the newborn preclusre piglet (Westrom et al. 1985). In addition a significant relationship was established between the consumption of milk proteins and the occurrence of disease (Scott 1990). This was marked with geographic variation (Dahl-Jorgensen et al. 1991). No significant differences were found between exclusively breastfed Icelandic children with diabetes and newly diagnosed diabetic children who received bovine milk-based formula and the non-diabetic children.
The frequency and extent of breastfeeding were comparable. While low disease occurrence among Icelandic children was not correlated with either total protein consumption (Elliot et al. 1999; Thorsdottir et al. 2000) or consumption of bovine serum albumin (Birgisdottir et al. 2002), it may be explained by significant lower levels of A1 and B beta-casein variants of Icelandic bovine milk compared to that in other Scandinavian countries (Thorsdottir et al. 2000). Similarly, Elliot (1999) has found that all analysed Icelandic milk samples have lower β-casein fraction A\(^1\) and B β-casein than other milk samples from Scandinavia. Per capita A\(^1\) and B β-casein and diabetes occurrence are significantly correlated (Elliot et al. 1999).

Unlike Scandinavian cattle breeds, Icelandic cattle have been isolated for about 1100 years leading to less frequent gene coding for A\(^1\) and B β-casein (Lien et al. 1999). This is dissimilar to β-casein A\(^1\) 60-67, bovine β-casein A\(^2\) and human β-casein that contain a proline amino acid at a corresponding position in their sequence. It is likely due to this that a proline peptide equivalent to BCM-7 cannot be formed upon digestion of human β-casein (Elliot et al. 1997). Furthermore, specific proliferation of T lymphocytes with bovine casein in those with T1ADM may account for the response of pancreatic β-cells cellular and humoral immune response to β-casein (Cavallo et al. 1996). This explains the specificity of the connection between the occurrence of diabetes and the consumption of a number of β-casein variants (Elliot et al. 1999). However, casein hydrolysate-based bovine milk formula was found to be highly effective in preventing autoimmune diabetes in NOD mice (Karges et al. 1997). Early reports on humans also indicate a protective effect of milk hydrolysates in humans as well (Akerblom et al. 2005).
People in developing countries are exposed to certain features of the affluent Westernised lifestyle including foods that find their way into their markets. Urbanisation is often accompanied by a shift in the availability and accessibility of foodstuffs. They often displace local and traditional food items and food habits resulting in a possible negative impact on health status (Williams 1999). Marked increase in the disease occurrence of T1ADM is occurring among the Jewish population with an exceptional increase among Yemenite Jews (18.5/100,000) compared to the overall occurrence rate among the Jewish population (5.7/100,000), and (2.9/100,000) among the Arab populations (Shamis et al. 1997).

Yemenite Jews have immigrated from voluntary isolated communities in underdeveloped countries (Shamis et al. 1997) to Israel which is a high-income Westernised country (Gross national income [GNI]: US$ 16,000/capita, 2002, Atlas method) (The World Bank Group 2004). Furthermore, it has been demonstrated that Yemenite Jews are genetically distinctive from other Israeli ethnic groups (Weintrob et al. 2001). The sharp increase in the disease occurrence among them may be attributed to changes in affluent economic status and Westernised lifestyle as a consequence of immigration (Shamis et al. 1997).

Worldwide economic disparities remain entrenched among and within different countries (Sen and Bonita 2000). Economic status under which populations live are among other conditions in determining the risks to which populations are exposed (World Health Organization Western Pacific Region 2002). The Kuwaiti population is approximately half that of Jordan, and has a high-income developing population (GNI per capita 16,340 US$, 2002) similar to that in the industrialised countries. In Kuwait, the mean and relative change in IMEB were higher by 2-fold, and 3-fold, respectively than that of Jordan, a severely-indebted lower-middle-income country (GNI per capita: US$ 1,760, 2002) (The World Bank Group 2004).
Milk protein consumption in Kuwait (14.51 ± 2.75 g/capita/day) was not only twice that of Jordanians, but also similar to that of affluent populations with substantial relative changes (36.49 ± 25.3). In Jordan MPC has increased from 3.6 g/capita/day in 1961 to 6.9 g/capita/day in 2001 (Beaudry 2004). Interestingly, while the disease occurrence among Kuwaitis has risen substantially from 3.95/100,000, (980-1981) (Taha et al. 1983) to 14.4/100,000 (1992-1993) (Shaltout et al. 1995), the disease occurrence in Jordanian children aged 14 years or less remained relatively unchanged. It has increased from 2.8/100,000 in 1992 to 3.6/100,000 in 1996 (AjlOUNI et al. 1999). It remains among the lowest in its region and matches that of the Arab population who live in neighbouring Israel. Arab-Israelites for instance are voluntarily isolated and live in poor communities similar to that the Yemenite Jews encountered prior to immigrating to Israel. Arab-Israelites continue to live in rural areas maintaining more traditional lifestyles that may explain the low disease occurrence among them (Shamis et al. 1997).

2.6 Conclusion

The results presented here agree with previous findings indicating a strong correlation between differences in milk consumption and the occurrence of autoimmune diabetes (Westrom et al. 1985; Scott 1990; Dahl-Jorgensen et al. 1991). It does seem reasonable to suggest that geographic variation in the disease occurrence among children aged 14 years or younger was associated with a worldwide variation in both mean and relative change in imported and consumed milk protein. Whether this variation is an indication of variation in the diabetogenicity of bovine milk proteins requires further longitudinal animal and human trials.
3 Baseline dietary profile of young Jordanian children with diabetes: Breastfeeding and early infant feeding practices

3.1 Abstract

Objective: A study was conducted to establish a dietary profile for diabetic and non-diabetic Jordanian children aged 14 year or younger by investigating the occurrence of breastfeeding and early infant feeding practices

Design and methods: Fourteen male ad 36 female diabetic children (DC), and 50 (22 male and 28 female) unrelated aged matched non-diabetic children (NDC) were identified by the National Centre for Diabetes, Endocrinology and Genetics, Jordan. All children were 14 years old or less, and children with known autoimmune disease were excluded. A constructed three-part questionnaire was developed. The percent HbA1c in blood was determined.

Results: Mean age at which children were diagnosed with diabetes was seven year with the majority being diagnosed in winter. No significant difference was found for body mass index. Signs of undernutrition were observed in both groups, whereas signs of obesity were seen in NDC. Dietary compliance in DC was poor (mean HbA1c 9.32%). Extended breastfeeding was equally prevalent in both groups to age 15 months. Sixty two percent of DC and 92% of NDC had bottle-feeding after 3 months of age, 8% of the NDC had no bottle-feeding. None of participants in both groups had solid foods before 3 months of age.
**Conclusion**: No significant association has been established for neither breastfeeding nor early infant feeding practices between both DC and NDC Jordanian children aged 14 years or less. Children in both groups were breastfed for at least some period of time with higher proportion of diabetic children receiving no bottle feeding in their infancy. Provisions for dietary compliance and nutritional counselling are necessary for young Jordanian children.
3.2 Introduction

In Jordan, the percentage of population aged below 15 years constitutes 37.8% of the overall four and a half million Jordanians (Department of Statistics 2004). In terms of health outcomes and access, however underutilised, inefficient and expensive, Jordan has one of the best performing health sectors in its region (Obermeyer and Potter 1991; Partners for Health Reform Plus 1997). Estimates indicate that more than 100,000 persons in the Middle East are prevalent with type 1 autoimmune diabetes (T1ADM) (Amos et al. 1997b). The estimated annual occurrence is around 6000; of these some 3000 are children below the age of 15 years.

In 1997, around 5,200 Jordanians had the disease; of them some 700 were children below 15 years old (Green 1997). The disease occurrence among Jordanian aged 14 years or less has increased from 2.8 per 100,000 children in 1992 to 3.6 per 100,000 children in 1996 (Ajlouni et al. 1999), nonetheless it still has among the lowest occurrence rates in the region (Shaltout et al. 1995; Kadiki et al. 1996; Soliman et al. 1996; Kulaylat and Narchi 2000). There is a dearth of information on both the epidemiology and the dietary profile of people with type 1 autoimmune diabetes in Jordan. The purpose of the present study was to embark on establishing baseline data on the dietary profile of Jordanian children with diabetes aged 14 years or younger in terms of their breastfeeding and early infant feeding practices.
3.3 Research design and methodology

3.3.1 The population sample

The cohort of this random case control prospective study consisted of 50 young Jordanian diabetic children (DC) who were identified during routine consultation visits to the outpatient’s diabetic clinic of The National Centre for Diabetes, Endocrinology, and Genetics (NCDEG), Jordan. Fifty non-diabetic Jordanian children were drawn from the general population and identified by Princess Rahmah Children’s Hospital (PRCH), and Al-Zahrawi Medical Laboratory, Jordan. The majority of participants in both groups live in Amman and Irbid, which account for more than a half of the Jordan population. Only children aged 14 years or less with T1ADM were included.

Diagnosis with diabetes was confirmed by the medical history of the diabetic children, including the presence of clinical symptoms and the need for insulin therapy. Participants were unrelated to those with diabetes or to each other and were age and gender matched with that of the participants of the control group. Ethical approval was granted by the Human Research Ethics Committee of the University of Western Sydney as well as by the authorities of all Jordanian participating institutions. All subjects, or their parents when appropriate, gave either written informed consent or a witnessed verbal consent when appropriate.
3.3.2 Survey tool

A constructed questionnaire (Appendix 10.1.3 and 10.2.3) was developed and ethically approved by all participating institutions. All subjects, or their parents when appropriate, gave either written informed consent (Appendix 10.1.2, and 10.2.2) or a witnessed verbal consent when appropriate. Participants and their parents received an information sheet explaining the research general background (Appendix 10.1.1, and 10.2.1). The questionnaire consisted of three main parts: the first part evaluates general nutritional status and sociodemographic characteristics, the second part examines general medical history related to diabetes mellitus, and the third assesses breastfeeding and early infant feeding practices prior to and after developing the disease.

3.3.3 Exclusion criteria

Children with any known form of rheumatic fever, rheumatoid arthritis, multiple sclerosis, and system lupus erythematosus at the time of surveying were not included in the study. These diseases may have affected the initiation of pancreatic autoimmune diabetes. To prevent genetic bias no more than one member of a nuclear family was included in either diabetic nor non-diabetic group.
3.3.4 **Estimation of validity and reliability**

A panel of paediatricians, nurses, university academics, parents, and day-care centres carers have reviewed the questionnaire to evaluate its content validity. After undertaking a pilot study and re-standardising the survey tool to meet the Jordanian cultural background the internal consistency reliability was estimated. Coefficient $\alpha$ was more than 81.2, which indicates that the test reliability must be high (Allen and Yen 1979).

3.3.5 **Estimation of glycosylated haemoglobin (HbA1c)**

The levels of HbA$_{1c}$ of diabetic children are routinely estimated during diabetic children routine visit to NCDEG. The percent HbA$_{1c}$ in human whole blood was determined using automated ion-exchange high performance liquid chromatography (Bio-Rad D-10®, Bio-Rad Labs, Inc, USA). Haemoglobin A$_{1c}$ was measured within the normal range less than 6.05%. The upper limit of normal (ULN) equals 6.1% (The Diabetes Control and Complications Trial Research Group 1993).

3.3.6 **Statistical analysis**

All data were coded, computer entered and analysed using the Statistical Package for Social Sciences (SPSS). Crude analysis comparing diabetic and control group was performed using parametric [independent T sample test for equality of means and analysis of variance (ANOVA)], and nonparametric [Chi-square ($x^2$)] statistic tests as appropriate. Under the assumption of normally distributed errors, the data fit a linear regression model.
Correlations between variables were computed by the bivariate correlations procedure. Phi and Cramer’s phi was calculated to measure the degree of association between variables derived from a table’s $x^2$ value. Based on values of a set of predictor variables, logistic regression model was used to estimate odds ratios (OR) with a 95% confidence interval (CI) for each of the independent variables in the model. Data were considered statistically significant at $P<0.05$.

### 3.4 Results

#### 3.4.1 General nutritional status and sociodemographic characteristics

Table 3-1 and Table 3-2 summarise the mean, standard deviation, percentages and statistical significance for the following variables: (1) age, weight, height, body mass index (BMI), birth order of the participants, and (2) age, education and occupation of the parents. There were no statistical differences between DC and NDC. Diabetic female children numbered more than males (72% and 28%, respectively). While the majority of diabetic males aged four to ten years, females were ten to fourteen years (43%, and 61% respectively, $P = 0.034$, phi = 0.36). No significant relationship was found for the age groups of the NDC within the diabetic children group ($P = 0.246$).

No statistical significant difference was found for the mean body mass index (BMI) in both DC and NDC. Percentile curves for body weight, height and body mass index of DC and NDC ranged between the 5th and 95th percentiles for mean age, respectively ($P = 0.323$). Mean BMI percentiles curves of the diabetic males and females were above the (25th and 50th percentile for mean age, respectively. $P = 0.064$), that was above the 75th percentile for both sexes in the NDC ($P = 0.739$).
Approximately 20% of both DC and NDC were below the fifth percentile curve. A minimum of 10 percent of children in both groups were overweight, and six percent of the NDC were obese (Figure 3-1). Mean haemoglobin A$_{1c}$ levels of DC was 9.32 (± Standard deviation [SD] 2.25, P = 0000), given a reference point of less than 6.05%; upper limit of normal (ULN) = 6.1%) (The Diabetes Control and Complications Trial Research Group 1993). The levels of mean haemoglobin A$_{1c}$ among individual levels have ranged from 6.2 to 14.7 %, only one diabetic child has a normal level.

Wide variation was observed for the occupation of the parents of participants in both groups. Fathers were mainly government employees, whereas mothers were often unemployed (i.e. homemakers). Variation for the education of the parents was also observed. Proportions of mothers in both groups who have had undergraduate and postgraduate degrees were higher than that of fathers (Table 3-2).
Table 3-1 Summary of the sociodemographic characteristics of diabetic and non-diabetic Jordanian children aged 14 years or less. Upper limit normal (ULN) = 6.1%. The results presented as mean ± standard deviation and as percentage within each group. Data were considered statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetic group (n = 50)</th>
<th>Control group (n = 50)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male: Female)</td>
<td>14:36</td>
<td>22:28</td>
<td>0.096</td>
</tr>
<tr>
<td>Child age (year)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>9.1 ± 3.67</td>
<td>8.80 ± 3.69</td>
<td>0.684</td>
</tr>
<tr>
<td>0 - ≤4</td>
<td>12%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td>&gt;4 - ≤10</td>
<td>36%</td>
<td>21%</td>
<td>0.010</td>
</tr>
<tr>
<td>&gt;10 ≤14</td>
<td>26%</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>Child weight (kg)</td>
<td>31.48 ± 14.73</td>
<td>32.12 ± 13.10</td>
<td>0.819</td>
</tr>
<tr>
<td>Child height (m)</td>
<td>1.32 ± 0.18</td>
<td>1.31 ± 0.16</td>
<td>0.753</td>
</tr>
<tr>
<td>Birth weight (kg)</td>
<td>2.96 ± 0.7</td>
<td>3.00 ± 0.20</td>
<td>0.698</td>
</tr>
<tr>
<td>Child body mass index (kg/m²)</td>
<td>16.90 ± 4.18</td>
<td>17.77 ± 4.54</td>
<td>0.323</td>
</tr>
<tr>
<td>Glycosylated haemoglobin A₁c (%)</td>
<td>9.32 ± 2.25</td>
<td>6.0 (ULN)°</td>
<td>0.000</td>
</tr>
<tr>
<td>Birth order</td>
<td>2.64 ± 1.79</td>
<td>2.90 ± 1.92</td>
<td>0.486</td>
</tr>
</tbody>
</table>
Table 3-2 Summary of the sociodemographic characteristics of the parents of diabetic and non-diabetic Jordanian children aged 14 years or less. The results presented as mean ± standard deviation and percentage within each group. Data were considered statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Diabetic group (n = 50)</th>
<th>Control group (n = 50)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother age (year)</td>
<td>35.43 ± 6.50</td>
<td>33.94 ± 7.78</td>
<td>0.306</td>
</tr>
<tr>
<td>Father age (year)</td>
<td>41.27 ± 9.15</td>
<td>41.80 ± 8.54</td>
<td>0.768</td>
</tr>
<tr>
<td>Mother education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤12 years</td>
<td>44 %</td>
<td>44 %</td>
<td>0.230</td>
</tr>
<tr>
<td>&gt;12 years</td>
<td>56 %</td>
<td>56 %</td>
<td></td>
</tr>
<tr>
<td>Father education</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤12 years</td>
<td>58 %</td>
<td>68 %</td>
<td>0.300</td>
</tr>
<tr>
<td>&gt;12 years</td>
<td>42 %</td>
<td>32 %</td>
<td></td>
</tr>
<tr>
<td>Mother occupation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>72 %</td>
<td>58 %</td>
<td>0.381</td>
</tr>
<tr>
<td>Labour</td>
<td>12 %</td>
<td>12 %</td>
<td></td>
</tr>
<tr>
<td>Government employee</td>
<td>14 %</td>
<td>24 %</td>
<td></td>
</tr>
<tr>
<td>Private professionals</td>
<td>2 %</td>
<td>6 %</td>
<td></td>
</tr>
<tr>
<td>Father Occupation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unemployed</td>
<td>4 %</td>
<td>4 %</td>
<td>0.139</td>
</tr>
<tr>
<td>Labour</td>
<td>24 %</td>
<td>8 %</td>
<td></td>
</tr>
<tr>
<td>Government employee</td>
<td>58 %</td>
<td>80 %</td>
<td></td>
</tr>
<tr>
<td>Private professionals</td>
<td>14 %</td>
<td>8 %</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3-1 Percentile curves for body weight, height and body mass index of diabetic children (DC) and non-diabetic (NDC) Jordanian children aged 14 years or less. The results presented as percentage within each group. Data were considered statistically significant at $P<0.05$.

![Percentile Curves](image)

### 3.4.2 General medical history related to diabetes mellitus

Median age at which children were diagnosed with diabetes was seven years (interquartile range (IQR): 2.9 to 10.0 years), mean age was 6.6 years (SD ± 3.8). Male and female DC were diagnosed at 5.4 and 7.11 years (SD ± 4.47 and ± 3.55 respectively, $p = 0.163$). Table 3-3 shows a statistically significant relationship between DC and NDC was established for both having a family member with diabetes ($P \leq 0.001$, phi = 0.50), and the type of diabetes they had ($P = 0.000$, phi = 0.50). No significant relationships were established for either the total number of relatives who had diabetes or the nature of that relationship.
Table 3-3 Family history of participating diabetic and non-diabetic young Jordanian children aged 14 years or less. The results presented as percentage within each group. Data were considered statistically significant at \(P<0.05\).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients group ((n = 50))</th>
<th>Control group ((n = 50))</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants having a family member with diabetes</td>
<td>68%</td>
<td>18%</td>
<td>(P=0.000)</td>
</tr>
<tr>
<td>Number of family members with diabetes the participants have</td>
<td>One person 60.6 %</td>
<td>100 %</td>
<td>(P=0.162)</td>
</tr>
<tr>
<td></td>
<td>Two persons 18.2%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Three persons 12.1%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; three persons 9.1%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Relationship to the family members</td>
<td>Sisters and brothers 24%</td>
<td>11%</td>
<td>(P \leq 1.00)</td>
</tr>
<tr>
<td></td>
<td>Parents 19%</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grandparents 57%</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>Type of diabetes family relatives had</td>
<td>Type 1 autoimmune diabetes</td>
<td>40%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>Type 2 (non-insulin-dependent)</td>
<td>60%</td>
<td>100%</td>
</tr>
<tr>
<td>Mothers having gestational diabetes</td>
<td>4.1%</td>
<td>0%</td>
<td>(P=0.149)</td>
</tr>
</tbody>
</table>
The findings presented here also demonstrated that approximately two thirds of DC were diagnosed in winter (January, September, October, November, and December). More than two thirds of the parents of the interviewees have indicated that prior to clinical onset of the diabetes, their children had suffered undiagnosed viral infections described as “colds, influenza, and gastric problems” that was accompanied with severe feverish signs. Approximately 38 percent of them were diagnosed at a routine hospital or primary health care centre.

Two thirds of the parents were educated on the signs and symptoms of the disease that was significantly affected by mothers education (P = 0.008). None of the DC participated in this study have any autoimmune disease, 12 percent of them had other chronic diseases (partial deafness, dwarfism, brucellosis, meningitis and hypothyroidism). One diabetic child had food allergy to fish and none of the participants had any chronic gastrointestinal diseases.

3.4.3 Breastfeeding and early infant feeding practices

Table 3-4 and Figure 3-2 exemplify that except for three diabetic children (P = 0.079), all other DC and NDC were breastfed for at least some period of time (P = 0.466) and also shows that however the proportion of the DC exclusively breastfed for less than three months was higher than that in the NDC (22% and 6% respectively). There was no significant relationship between the DC and NDC for either the duration of exclusive breastfeeding (BRF) for three months or less (94% and 100% respectively, P = 0.466) or for the duration of sustained BRF (72% and 94%, P = 0.672 respectively).
The duration of BRF for less than three months of life was significantly correlated with
the age and the occupation of the mothers of the participants (r = - 0.570 and 0.616, P
<0.05, respectively). The mean duration of sustained breastfeeding in the diabetic and
non-diabetic children was 15.94 ± 13.09 and 15.06 ± 4.89 (P = 0.672), respectively. This
was correlated with the birth order of the participant (r = 0.237, P <0.05).

Diabetic children who had no bottle-feeding (BOF) at any age were significantly different
from the NDC who had no bottle-feeding at any age (P= 0.041). Nonetheless, there was
no significant relationship between both groups in neither introducing BOF at age
younger than three months (P = 0.130) nor in the duration of continued BOF (P = 0.672).
None of the NDC had any solid foods prior to three months of age. A statistically
significant relationship between DC and NDC was found for the introduction of
supplementary solid foods at the age of nine months or less (P =0.000, Cramers’phi =
0.436). Wheat was the most supplementary food introduced to NDC and rice was the
most common supplementary food DC had (P = 0.020, Cramer’s phi= 0.279). The
introduction of solid foods after nine months of age was significantly correlated with the
gender of the participants (r = 0.841, P <0.01).

Bovine milk-based formulae are widely used in nurturing both DC and NDC (P = 0.295).
Neither DC nor NDC had received soymilk. Few parents had switched between bovine-
milk based formula and fresh bovine milk as the child develops diabetes (P = 0.295).
Figure 3-3 illustrates that a third of the parents of DC and NDC prepared their children’s
liquid bottle-milk feeds by dissolving canned infant milk powder in lukewarm water and
then boiling together. This was not significantly related with those who prepared it by
mixing canned infant milk formula with preboiled water (P = 0.907).
Table 3-4 Breastfeeding and early infant feeding practices in young Jordanian diabetic and non-diabetic children aged 14 years or less. Results presented as percentage within each group and mean ± standard deviation. Data were considered statistically significant at P<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients group (n = 50)</th>
<th>Control group (n = 50)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Exclusive Breastfeeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No breast feeding</td>
<td>6%</td>
<td>0</td>
<td>P =0.079</td>
</tr>
<tr>
<td>&lt;1 week</td>
<td>4%</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&gt;2 weeks – 1 month</td>
<td>4%</td>
<td>0</td>
<td>P =0.466</td>
</tr>
<tr>
<td>&gt;1 month – 3 months</td>
<td>14%</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td><strong>Continued breastfeeding</strong></td>
<td>(72%)</td>
<td>(94%)</td>
<td>P = 0.672</td>
</tr>
<tr>
<td>For &gt;3 months</td>
<td>15.94 ± 13.09</td>
<td>15.06 ± 4.89</td>
<td></td>
</tr>
<tr>
<td><strong>Initiation Of bottle-feeding</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No bottle-feeding</td>
<td>8%</td>
<td>0</td>
<td>P = 0.041</td>
</tr>
<tr>
<td>&lt;1 week</td>
<td>16%</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&gt;1 – 2 weeks</td>
<td>2 %</td>
<td>2 %</td>
<td>P = 0.130</td>
</tr>
<tr>
<td>&gt;2 weeks – 1 month</td>
<td>8 %</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>&gt;1 – 3 months</td>
<td>4 %</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>&gt;3 months</td>
<td>(62%)</td>
<td>(92%)</td>
<td>P = 0.672</td>
</tr>
<tr>
<td></td>
<td>13.09 ± 7.18</td>
<td>11.17 ± 3.36</td>
<td></td>
</tr>
<tr>
<td><strong>Type formula introduced</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine milk-based</td>
<td>90%</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>Fresh bovine milk</td>
<td>2%</td>
<td>2%</td>
<td>P = 0.295</td>
</tr>
<tr>
<td><strong>Introduction of supplementary solid foods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 months</td>
<td>16%</td>
<td>0%</td>
<td>P =0.000</td>
</tr>
<tr>
<td>3-6 months</td>
<td>30%</td>
<td>66%</td>
<td></td>
</tr>
<tr>
<td>6-9 months</td>
<td>46%</td>
<td>26%</td>
<td></td>
</tr>
<tr>
<td>&gt; 9 months</td>
<td>(8%)</td>
<td>(8%)</td>
<td>P = 0.132</td>
</tr>
<tr>
<td></td>
<td>14.25 ± 4.50</td>
<td>10.25 ± 0.96</td>
<td></td>
</tr>
<tr>
<td><strong>Solid foods introduced</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>62%</td>
<td>84%</td>
<td></td>
</tr>
<tr>
<td>Rice</td>
<td>30%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>8%</td>
<td>0%</td>
<td>P = 0.020</td>
</tr>
</tbody>
</table>
Figure 3-2 Proportion of Jordanian diabetic children (DC) and non-diabetic children (NDC) aged 14 years or less who had breastfeeding (BRF), bottle-feeding (BOF) and exposed to solid foods supplements at three months (m) of age or more. The results presented as percentage within each age group. Data were considered statistically significant at \( P<0.05 \). No BRF (\( P=0.079 \)), BRF >3 months (\( P=0.672 \)), no BOF (\( p=0.041 \)), BOF >3 months (\( p=0.672 \)) and solid s >3 months (\( P=0.000 \)).
Figure 3-3 Home heat processing procedures that parents used to prepare their infants liquid bottle milk feeds both diabetic children (DC) and non-diabetic (NDC) Jordanian children. The results presented as percentage within each group. Data were statistically insignificant (P<0.907).
3.4.4 Risk of having the disease

The logistic regression analysis yielded that BRF and BOF at least for some period was associated with a smaller risk of developing diabetes (OR 0.560; 95% CI 0.199; 1.571, P=0.432). Introducing BOF both prior to and after three months of age to children breastfed at least for some period of time was associated with a relatively greater risk of having the disease (OR 1.56; 95% CI 0.792; 3.067 P=0.091, and OR 0.926; 95% CI 0.845; 1.041, P=0.342 respectively). This was similar when BOF was initiated at an age less than three months to children breastfed for less than three months (OR 1.423; 95% CI 0.915; 2.212, p=0.780). The risk was relatively lower if BOF was introduced to children breastfed for more than three months of age (OR 0.999; 95% CI 0.995; 1.003, P=0.278).

Regardless of the duration of BRF, introduction of supplementary foods was associated with nearly the same risk of having the disease. Introduction of wheat or rice to children breastfed for both less and more than three months was associated with relatively the same risk (OR 0.998; 95% CI 0.697; 1.430, P=0.320 and OR 0.956; 95% CI 0.914; 0.999, P=0.430). Nonetheless, the risk was higher with the age at which BOF was introduced, particularly at an age less than three months compared to introduction at more than three months of age (OR 1.362; 95% CI 0.769; 2.413, P=0.850 and 0.896; 95% CI 0.825; 0.973, P=.462). The way by which parents prepared bottle-milk feeds and introducing BOF prior to three months of age was associated with a greater risk of having the disease (OR 1.884; 95% CI 0.838; 4.239, P=0.820). The risk was lower when introduced at more than three months of age (OR 0.962; 95% CI 0.915; 1.012, P=0.294).
3.5 Discussion

The first and only study on the epidemiology of type 1 autoimmune diabetes was in 1999 (Ajlouni et al. 1999). This study, to the best of our knowledge, constitutes the first to establish a primary baseline data on the dietary profile of Jordanian children with diabetes aged 14 years or less by studying their breastfeeding and early infant feeding practices. Unlike type 2 (non-insulin-dependent) diabetes, type 1 autoimmune diabetes, at the present time, does not constitute a health problem in Jordan (Ajlouni et al. 1998a). Jordan faces critical challenges regarding high population growth rates that will double the population in 24 years (Department of Statistics 2004). It is projected that the number of children with diabetes will increase 10-fold by 2028, given the current disease occurrence rate and if the crude death rate remains unchanged.

The structure of Jordan population is complex and shaped by both historical and cultural factors (Nabulsi et al. 1997). Jordan is a severely-indebted lower-middle-income country (GNI per capita 1760, 2002) (The World Bank Group 2004). More than 70% of the Jordanian population are urban dwellers with an average 5.7 persons per household. More than 37% are children below 15 years of age (Department of Statistics 2004). The Jordan population is endogamic involving inbreeding subunits with a wide range of genetic diversity (Nabulsi 1995; Nabulsi et al. 1997).

Jordan is a north of the equator Asian country, at low risk population and fits the worldwide geographic distribution model of the disease (Karvonen et al. 1993). It has one of the lowest disease rates. The age-specific disease occurrence rate was the highest among Jordanian children aged 10-14 years and the lowest was among children less than four years of age (5.5 and 1.3 per 100,000 respectively) without gender differences (Ajlouni et al. 1999).
In the early years of the twenty first century, six percent of the general Jordanian population were undernourished. Five percent of children aged five years are underweight. Eight percent of infants were born with low-birth weight (United Nations Development Programme 2003; UNICEF 2004). The distribution of anaemic children, haemoglobin less than seven, has risen from 0.13% in 2003 to 0.22% in 2004. For anaemic children of 10 to <11 haemoglobin the distribution has risen from 24.7% in 2003 to 25.6% in 2004 (Ministry of Health Jordan 2005) with signs of protein-energy malnutrition among infants (Hijazi 1974).

A study on Jordanian children has shown that the height-for-age values fluctuated between the 5th and 10th percentiles for both males and females. The body weight-for-age was just above the 25th percentile for males, whereas it fluctuated between the (25th and 50th percentiles) for females. Body mass index of male children were just above the 50th percentile and for females it varied between the 50th and 75th percentiles until 13.5 years of age (Hasan et al. 2001). Data on BMI were compared with the American percentile curves of body mass index (Hammer et al. 1991) given the unavailability of standardised national BMI percentile curves and the implications of ethnic differences (Hosseini et al. 1999; Denney-Wilson et al. 2003; Karasalilhoglu et al. 2003).

Jordan provides free healthcare and nutrition programmes for children, including free insulin injections instituted by The Monarch of Jordan, King Abdullah II in 2000 (Jordan Times 1999). The World Food Program-Assisted Project provides school children with approximately 690 calories per day (Hijazi and Adbulatif 1986). In addition, the School Nutrition Program (SNP) also provides a daily fortified mid-morning snack containing essential vitamins for approximately 54,000 school children to schools in remote underprivileged areas (Jordan Times 2004).
In addition to specialised medical services rendered by the National Centre for Diabetes, Endocrinology and Genetics, a state-of-art centre was established in 1996 (The National Center for Diabetes 2005). The Jordan River Foundation (JRF), a leading non-government organization, chaired by Her Majesty the Queen has recently launched a three-year strategic plan focusing on the Jordan River Children’s Program as one of the Queen Rania Centre for the Family and Child major agendas on child safety and early childhood development issues (Al-Abdullah 2004).

Obesity seems to affect more than half of Jordanian youths above 25 years (Ajlouni et al. 1998b), while females have a propensity to develop obesity after puberty (Hasan et al. 2001). Obesity and rapid linear growth are considered as risk factors for T1ADM in children as reported in most industrialised countries (Hypponen et al. 2000). The occurrence of childhood type 2 diabetes is now prevailing among children as well (Drake et al. 2002). The increased occurrence of childhood obesity is of concern and requires preventative measures (Gahagan et al. 2003). Early identification of childhood obesity is a key factor in preventing obesity and its health related complications in later adulthood (Leong and Wilding 1999; Howdle and Wilkin 2001; Al-Domi et al. 2005).

Average HbA$_{1c}$ levels more than two percent above the upper limit of normal (6.1%) indicate considerable risks for microvascular complications (The Diabetes Control and Complications Trial Research Group 1993; Davidson et al. 1999). The level of HbA$_{1c}$ was elevated well above the upper normal limit in 98% of the DC Jordanian children and was above the 10% level in one third of them. Haemoglobin A$_{1c}$ levels indicate how blood glucose is handled in the long-term rather than one instance of time (Davidson 2001).
Poor dietary habits and sedentary lifestyle as well as poor dietary counselling are most likely the main reasons why diabetics are not achieving the recommended HbA1c levels. Variation in physician expectations and the availability of licensed dieticians and nutrition counselling practitioners may contribute significantly to dietary compliance. In Jordan, nutrition and nutrition counselling are not recognised as medical professions rather nutrition and dietetics practitioners are often graduates of either food sciences or general public health with major in human nutrition disciplines. Furthermore, the patient load for each nutrition practitioner is burdensome. An interdisciplinary and collaborative team of which the person with diabetes is the main focus is the most suitable team to undertake (Al-Domi et al. 2005).

The rate of breastfeeding in the developing countries was 52% in 2001 (UNICEF 2001). In Jordan, the percentage of infants exclusively breastfed was 67% and 23% (0-4 and less than 6 months, respectively) and 70% of children six to nine months of age were breastfed with complementary food, whilst at 20 to 23 months, 12% were still BRF. A minimum of 90% of all mothers initiate some BRF and 27% of them initiate BRF within the first hour after birth (UNICEF 2004; Ministry of Health Jordan 2005). The reduction of BOF and improved BRF practices may save an estimated 1.5 million children each year. However, it was reported that only 16 countries, Jordan among them, have achieved full compliance with the code of baby formula marketing (UNICEF 1997). Jordan has adopted the Baby-Friendly Hospital Initiative in 1993 with three existing baby-friendly facilities (UNICEF 2005).
The hypothesis of an inverse correlation between the protective effect breastfeeding may have on the development of T1ADM, the implication of the early introduction of BMP at early age with the disease aetiopathogenesis has undergone worldwide extensive investigation, and it remains widely controversial. Meta analysis of nearly thirty retrospective studies have failed to establish a strong relation between the protective effect of breastfeeding and age when introducing BMP (Gerstein 1994; Norris and Scott 1995). The only prospective clinical dietary intervention trial on humans has recently suggested a possible role dietary intervention may have on the initiation of pancreatic autoimmunity in genetically susceptible infants (Akerblom et al. 1999; Akerblom et al. 2005).

While a Sardinian case-control study has demonstrated that the risk of developing the disease in Sardinian children who were not BRF was insignificant (Meloni et al. 1997), a Swedish and Norwegian time-series (1940-1980) study has ascertained an inverse relationship between the duration of breastfeeding and the development of T1ADM (Borch-Johnsen et al. 1984). On the other hand, studies have indicated that independent of the duration of BRF, young age at introducing BMP is the most vital risk factor (Verge et al. 1994; Gimeno and de Souza 1997). Regardless of the HLA genotypes, two prospective studies, the Australian AudDiab study (Couper et al. 1999) and the German BABYDIAB study (Hummel et al. 2000), were not able to establish that breastfeeding may have a protective effect on the development of the disease. These findings remain controversial and discrepant with strong indications. It indicates that beta cell destruction is a complex process, which is affected by inert-individual variations as well as variation between low and high-risk populations.
3.6 Conclusion

Regardless of their group, there was no significant relationship between DC and NDC for the duration of either BRF, or in the instigation of BOF or the introduction of supplementary foods. Breastfeeding in Jordan is common and is usually extended for a minimum of one year for the majority of children. A higher proportion of diabetic children received no bottle-feeding in their infancy. Dietary compliance in Jordanian children with diabetes was poor with signs of undernutrition in both groups as well as signs of obesity in controls. In Jordan, for better nutrition compliance there does seem a need for better dietary monitoring and professional dietary counselling programmes for both diabetic and non-diabetic children as well as establishing a national diabetes registry and developing national dietary guidelines.
4 Proportion of ABO and Rh blood systems in young Jordanians with diabetes: A sign of protective effect

4.1 Abstract

**Objective:** A study was conducted to examine whether ABO and Rh (D) blood groups are associated with having type 1 autoimmune diabetes in young Jordanian children.

**Design and methods:** diabetic children (DC) and their unrelated aged matched non-diabetic children (NDC) were identified. All children were 14 years old or less. The ABO and Rh (D) phenotypes of the participants were determine by slide method at room temperature. The specifications of the manufacturer were maintained. Positive and negative controls were tested in parallel with each batch of tests.

**Results:** Statistically significant relationships were found for ABO blood group of both DC and NDC (P = 0.002). The most frequent blood group in DC and NDC was O+ blood group (54% and 38% respectively) followed by A+ blood group (30% and 22%, respectively) and B+ blood group (16% and 8%, respectively). None of the DC had AB+, A- or O- blood group. All DC were Rh (D) positive, whereas 80% of NDC were Rh (D) positive (P ≤ 0.001), 8% of NDC of Rh (D) negative had B blood group.

**Conclusion:** Blood group O+ was associated with a greater risk of developing diabetes. Whether certain blood group may confer a protective effect from diabetes or not requires further investigation on the secretor status of humans with type 1 autoimmune diabetes and viral infections.
4.2 Introduction

A specific complex of antigens present on red blood cells genetically determines blood groups characteristics. Immunological characteristics determine and classify the differentiation of blood by type (Watkins and Morgan 1959; Morgan and Watkins 2000). Although there are more than 25 major blood group systems, a minimum of 270 red cell phenotypes and more than six hundred red cell membrane antigens are recognised (Green 1989; Garratty et al. 2000; Hughes-Jones and Gardner 2002), the ABO blood groups system, first introduced in 1901, remains the most used system.

Studies have demonstrated the implication of different blood group phenotypes in disease pathogeneses. The ABO and Rhesus [Rh (D)] blood groups have been implicated in increased susceptibility to certain diseases (Blackwell 1989) for instance, *Helicobacter pylori* and the increased risk of peptic ulcer (Alkout et al. 1997; Alkout et al. 2000), haemolytic uremic syndrome and *Escherichia coli* (Blackwell et al. 2002), elevated serum antibody titre levels to *vibrio cholera* (Swerdlow et al. 1994), carcinomas (Su et al. 2001) and infertility in women (Lurie et al. 1998).
Studies on maternal-child blood group incompatibility (Dahlquist and Kallen 1992), type 2 (non-insulin-dependent diabetes) (Sidhu et al. 1988; Qureshi and Bhatti 2003) and T1ADM (Kanazawa et al. 1983; Pontiroli et al. 1984) have demonstrated associations for the distribution of the ABO blood groups in diabetics different to the general population. Differences in the frequency of ABO and Rh blood groups indicate racial and ethnic variations in individuals indigenous to various parts of the world (May and du Toit 1989; Wagner et al. 1995; Falusi et al. 2000; Chiaroni et al. 2004). The worldwide paucity of studies on type 1 autoimmune diabetes particularly in young Jordanian children has encouraged the researcher to study the frequency of ABO and Rh (D) blood groups among them.

4.3 Research design and methodology

4.3.1 The population sample

The cohort of this study consisted of 50 young Jordanian diabetic children (DC) (36 females and 14 males) identified by The National Centre for Diabetes, Endocrinology and Genetics (NCDEG), Jordan, and 50 (28 females and 22 males) unrelated, age and sex matched non-diabetic children (NDC) were identified by Princess Rahmah Children’s Hospital (PRCH)) and Al-Zahrawi Medical Laboratory, Jordan. Ethical approval was granted by the Human Research Ethics Committee of the University of Western Sydney as well as by the authorities of all Jordanian participating institutions. All subjects, or their parents when appropriate, gave either written informed consent or a witnessed verbal consent when appropriate.
4.3.2 Determination of ABO and Rh blood groups

A licensed phlebotomist drew (5ml) venous blood samples. Specimens were tested immediately following collection. Anti-A, anti-B and anti-D monoclonal murine IgM blood grouping reagents obtained from Bioscot®, (Serologicals Ltd, UK) were used to determine the ABO and Rh (D) phenotypes of the participants by slide method at room temperature. Manufacturer’s specifications were maintained and positive and negative controls were tested in parallel with each batch of tests.

4.3.3 Statistical analysis

All data were coded, computer entered and analysed using the Statistical Package for Social Sciences (SPSS). Nonparametric [Chi-square \( \chi^2 \)] statistic tests used as appropriate. Phi and Cramer’s phi were calculated to measure the degree of association between variables derived from a table’s \( \chi^2 \) value. Based on values of a set of predictor variables, the logistic regression model was used to estimate odds ratios (OR) with 95% confidence interval (CI) for each of the independent variables in the model. Data were considered statistically significant at P<0.05.
4.4 Results

Figure 4-1 shows statistically significant relationships found for ABO blood groups of both DC and NDC (P = 0.002, Cramer’s phi = 0.44). The most frequent blood group in DC and NDC was O⁺ blood group (54% and 38% respectively) followed by A⁺ blood group (30% and 22%, respectively) and B⁺ blood group (16% and 8%, respectively). None of the DC had AB⁺, A⁻ or O⁻ blood group. All DC were Rh (D) positive, whereas 80% of NDC were Rh (D) positive (P ≤ 0.001, phi = 0.33), 8% of NDC of Rh (D) negative have B blood group.

Figure 4-1 Distribution (%) of the ABO and Rh (D) blood groups of young diabetic children (DC) and non-diabetic children (NDC). The results presented as percentage within each group. ABO blood groups and Rh (D) positive of both DC and NDC were statistically significant (P=0.002 and P≤0.001 respectively).
Table 4-1 shows the gender variation of ABO and Rh (D) blood groups among participants with regard to their gender. A significant relationship was found for the ABO blood groups between diabetic and non-diabetic females. No significant relationship was found for ABO or Rh (D) blood groups between diabetic and non-diabetic males and diabetic and non-diabetic females.

Table 4-1 Comparison between the ABO and Rh (D) blood groups of young male and female diabetic and non-diabetic children. The results presented as percentage within each group. Data were considered statistically significant at P<0.05.

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<th>A+</th>
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<th>B+</th>
<th>AB+</th>
<th>O+</th>
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<td><strong>Diabetic Males</strong></td>
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<td>(n = 14)</td>
<td>28.5%</td>
<td>0%</td>
<td>14.5%</td>
<td>0%</td>
<td>57.0%</td>
<td>0.0%</td>
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<tr>
<td><strong>Non-diabetic Males</strong></td>
<td>23%</td>
<td>9.0%</td>
<td>9.0%</td>
<td>9.0%</td>
<td>41.0%</td>
<td>9.0%</td>
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<td>(n = 22)</td>
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<tr>
<td><strong>Diabetic Females</strong></td>
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<tr>
<td>(n = 36)</td>
<td>30.5%</td>
<td>0.0%</td>
<td>16.5%</td>
<td>0.0%</td>
<td>53.0%</td>
<td>0.0%</td>
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<tr>
<td><strong>Non-diabetic Females</strong></td>
<td>21.5%</td>
<td>10.7%</td>
<td>7.0%</td>
<td>14.1%</td>
<td>%36</td>
<td>10.7%</td>
<td>0.008</td>
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<td>(n = 28)</td>
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The logistic regression analysis has shown that ABO and Rh (D) blood groups were associated with a greater risk of developing diabetes (OR 1.022; 95% CI 0.884; 1.183). Blood groups were also associated with relatively low risk of developing the disease with regard to the gender (OR 0.978; 95% CI 0.909; 1.054).
4.5 Discussion

The present study, to the best of the researcher’s knowledge, constitutes the first data on ABO and Rh (D) blood groups of diabetic Jordanian children aged 14 years or less. The findings of the present study constitute baseline data for both diabetic and non-diabetic Jordanian children aged 14 years or less, given the unavailability of reliable records for the distribution of the blood groups in the general population. The ABO blood groups system is the most important and the most common human alloantigen system in blood transfusion (Yamamoto et al. 1992).

Genetic role in the determination of ABO and Rh (D) blood groups system (Green 1989; Garratty et al. 2000; Hughes-Jones and Gardner 2002) exhibited extensive variation in the distribution of ABO and Rh (D) blood groups in different populations (May and du Toit 1989; Wagner et al. 1995; Falusi et al. 2000; Chiaroni et al. 2004). The main ABO alleles that create familiar A, B, AB and O blood groups exhibit broad sequence heterogeneity and extensive sequence variation in both the coding and non-coding region of the gene, given that a minimum of 70 ABO alleles are reported (Yip 2002).

Studies on maternal-child blood group incompatibility (Dahlquist and Kallen 1992), type 2 non-insulin-dependent diabetes (Sidhu et al. 1988; Qureshi and Bhatti 2003) and type 1 insulin-dependent diabetes (Kanazawa et al. 1983; Pontiroli et al. 1984; Karamizadeh and Amirhakimi 1996) have demonstrated associations for the distribution of the ABO blood groups in diabetics different to the general population. An increased risk of developing type 1 autoimmune diabetes due to maternal-child blood group incompatibility was established in Swedish diabetic children aged 14 years or less (Dahlquist and Kallen 1992).
Studies on type 2 diabetes mellitus showed inconsistency in the distribution pattern of ABO and Rh (D) blood groups. While the distribution of B blood group was elevated among Pakistanis (Qureshi and Bhatti 2003), the proportion of A, AB and Rh(D) positive blood groups were increased among Indians with the disease (Sidhu et al. 1988), whereas no significant difference was established in Iranians with diabetes (Karamizadeh and Amirhakimi 1996). These differences in the frequency of ABO and Rh blood groups indicate racial and ethnic variations in persons indigenous to various parts of the world (May and du Toit 1989; Wagner et al. 1995; Falusi et al. 2000; Chiaroni et al. 2004).

Significant relationships were found for the prevalence of fast acetylator phenotype in diabetic adults who had blood group B, given fast acetylator prevalence was significantly higher in diabetic children than adults and non-diabetics (Pontiroli et al. 1984). While, no associations were found for MN, KIDD and Lewis blood group systems in both diabetic adults and diabetic children grouped according to insulin dose administered, it was statistically significant for the ABO blood groups in patients receiving higher insulin doses (Kanazawa et al. 1983).

Numerous studies have challenged the importance of ABO blood groups. For instance, the increased binding of Helicobacter pylori to epithelial cells and increased risk of peptic ulcer (Alkout et al. 1997; Alkout et al. 2000), duodenal and gastric ulcers (Mentis et al. 1991), haemolytic uremic syndrome caused by Escherichia coli O157 (Blackwell et al. 2002), diarrhoea and heat labile enterotoxin producing Escherichia coli (Black et al.) and elevated serum antibody titre levels to vibrio cholera (Swerdlow et al. 1994) are associated with a higher proportion of people of O blood group phenotype.
The ABO histo-blood system includes three antigens (ABH). Unlike people of A, B and AB groups can convert the H antigen into A or B antigens, people of O blood group having guanine (258) deleted in the O gene produce an inactive protein incapable of converting the H antigen (Yamamoto F 1990). The prevalence of *Helicobacter pylori* in non-secretor people with duodenal ulcer was significantly higher than that of non-secretor people who had gastric ulcer (Mentis et al. 1991).

The majority of diabetic children who participated in this study were diagnosed in winter and the majority have encountered a viral infection preceding the clinical onset of autoimmune diabetes. An association between secretor status and the susceptibility to develop type 1 autoimmune diabetes with elevated proportion of non-secretors among them was also demonstrated (Blackwell et al. 1987). Given the heterogeneity in the expression of glycoconjugates associated with different histo-blood group ABO (Yamamoto F 1990; Yamamoto et al. 1992; Yamamoto et al. 1993), it was established that secretor gene shows signs of protective effect particularly in immunocompromised people (Blackwell 1989).

### 4.6 Conclusion

Blood group O+ is significantly predominant in young Jordanian children with diabetes and is associated with a greater risk of developing diabetes. A higher proportion of NDC had Rh (D) negative blood groups with gender variation. Whether certain blood groups confer a protective effect from diabetes requires further investigations on the secretor status of humans with type 1 autoimmune diabetes.
5 Evidence on the heterogeneity of native, modified and heat processed milk proteins: A spectral profile

5.1 Abstract

Objective: Bovine milk proteins have been widely implicated in the development of type 1 autoimmune diabetes in early infancy. Solubilized proteins have distinctive absorption properties which can be quantitated independently devoid of extensive procedures.

Design and methods: Human breast milk, bovine-based liquid, and reconstituted adult and infant bovine milk-based formulas were cooled (4 °C/20 min) and separated at 15,000 rpm for one hour at 4 °C. to replicate home and industrial heat processes; milk proteins were heat processed by both water bath heat treatment (40-100 °C/5-60 min) and/or hot plate heat treatment (63 °C/15-30 min). Absorbance spectra of native and treated milk proteins were screened in the ultraviolet region, and the pH was recorded at 25 °C.

Results: A unique identification standard curve was established for each of the tested proteins. The linear range of the assay for human-breast milk, bovine serum albumin and the rest of the tested proteins was 20-100 mg/ml, 1 mg/ml, and 10 mg/mL respectively. The absorption maxima of human-breast milk proteins, BSA, and the rest of the tested milk proteins fall into three maxima in the ultraviolet region (λ270nm, λ275 nm, and λ270-λ275 nm; respectively). Changes in the absorption maxima of the tested milk proteins subjected to various heat treatments were detectable at temperatures as low as 40 °C for 30 min, and were concentration dependent. Human breast-milk has a neutral pH (7.2), whereas the vast majority of the tested bovine milk proteins were slightly acidic.
**Conclusion:** The findings of the present study have ascertained the heterogeneity of both human breast milk and bovine milk protein solutions. Different native, modified and heat processed milk protein solutions have exhibited a distinctive spectral profile with various intensities. The present assay was rapid, sensitive, and reproducible.
5.2 Introduction

Although bovine milk proteins have undergone an intensive and extensive investigation as a putative environmental factors implicated in the destruction of pancreatic beta cell, no causal links have been established and findings remain widely controversial and discrepant. Milk proteins are widely heterogeneous (Goff and Hill 1993) and that discrepancy in the findings indicates variations in diabetogenicity of milk proteins (Thorsdottir et al. 2000).

Heat processing is most commonly used in canned bovine milk-based foods processing technologies and is attributing to some undesirable and possibly severe quality defects to milk products including the denaturation of its protein (Pisecky 1997), which depends among other factors on heating temperature (Bertrand-Harb et al. 2002). Heat processing often causes protein antigens to aggregate, which affect both the immunogenicity (Harlow and Lane 1988) as well as physical nature of the proteins (Saperstein and Anderson 1962).

Complex proteins have distinctive absorbance properties and can be quantitated independently devoid of extensive purification procedures (Kaplan and Pesce 1996). The measurement of the light absorbance of substances is ordinary to many basic scientific and clinical research applications. It is one of the most widely used analytical techniques in determining the concentration of almost any molecule or class of molecules, studying the biochemical properties of various molecules (Lowry et al. 1951; Bradford 1976; Smith et al. 1985; Stoscheck 1990; Kaplan and Naito 1996) and studying the degree of denaturation of milk proteins (Garcia-Hernandez et al. 1998).
The spectral characteristics of milk and milk products influence their appearance. This has provided the bases for many rapid indirect methods of analysis (Goff and Hill 1993; Goff 2004). The purpose of the present study was to verify the diversity of milk proteins as well as to identify changes that modification and thermal processing may cause to milk proteins by studying changes in their spectral and chromatographic profiles.

5.3 Methods

5.3.1 Milk samples

Human breast milk (HBM) donated from a volunteer mother was collected over 24 hours and kept at (4ºC), raw whole pooled bovine milk (RWPBM) was obtained from the University of Western Sydney Dairy Farm and commercial whole pasteurised and homogenised fresh bovine milk (PHFBM) was obtained from local markets.

In addition, six canned bovine-based milk formulae were analysed. Three adult formulae (not suitable for infants). (Adult H (skim generic, Australia), Adult J (skim; Australia) and Adult D (full cream; Australia) were analysed. Two canned whey dominant bovine-based starter infant formulae (ISFK; New Zealand and ISFS; USA) and one canned enzymatically hydrolysed whey protein infant formula (ISFN; Germany), and bovine serum albumin (BSA) (Sigma, USA) and bovine insulin (BI) (Sigma-Aldrich, Australia) were also analysed. pH was recorded at 25ºC.
5.3.2 Reconstitution, separation, and modification of milk protein solutions

Milk powders, BSA and BI were reconstituted in double deionised ultra-filtered water (0.18 µm) at room temperature (24°C). The separation process of whole milk solutions was standardised; the best separation of HBM, and liquid and reconstituted bovine milk protein solutions (BMPs) was achieved by precooling milk solutions to 4°C for 20 minutes followed by centrifugation at 25,000 g for 60 minutes at 4 °C (70 mm diameter rotor, Beckman J2-HS).

Supernatant protein layers were gently aspirated, sterile-filtered through a 0.22 µm filter (Millipore, UK) and stored frozen at -70°C. The wet weight of fat and precipitants was recorded. One milliter of BSA (1mg/ml) was modified with an equal volume of BI (1mg/ml) and then 0.2 – 0.8 mg/ml of BSA was modified with one minus 0.2 to 0.8 mg/ml BI alternatively. The modification of BSA with BI was strictly carried out under aseptic conditions under room temperature (RT) (17°C/24h, pH 7.4) and under physiological conditions (37°C/24h), both were shaken continuously at 25 round per minute (rpm).

5.3.3 Water bath heat processing

Sterile-filtered BMPs, native BSA and modified BSA with BI (m-BI-BSA) were subjected to various water bath heat treatment at temperatures ranging from 40 to100°C and held for five to sixty minute. Treatments were carried in duplicates and strictly under the same the conditions. Samples of milk protein solutions (10mg/ml) were placed in heat-resistance thin-wall test tubes (Pyrex 18 x 150mm).
Sealed test tubes were submerged in a preheated water bath at a constant temperature at different preset times. One minute was allowed to warm the sample to the required temperature. Heat processed BMPs were cooled immediately to zero °C by submerging them in ice cubes mixed with water and table salt for 15 minutes. Cooled samples were stored frozen at -70°C for 24 hours prior to analysis.

### 5.3.4 Hot plate heat processing

To reach a constant temperature of 63°C, Sterile-filtered BMPs (10mg/ml) were preheated in sealed thin-walled heat-resistant beakers (Pyrex 80 x 100 mm) at 100°C and held for 30 seconds. Preheated samples duplicates were transferred immediately to a preheated hot plate yielding a constant temperature of 63°C and held for 15 and 30 minutes. Heat processed MPs cooled immediately as described above and stored at -70°C for 24 hours before analysis.

### 5.3.5 Absorbance spectrum

The absorbance maximum of HBM, and native and heat processed BMPs was measured in the ultraviolet region of the spectrum using a dual beam Multiskan® Spectrum spectrophotometer (Thermo Electron Corp., SkanIt software research edition). It has both the sample and the reference beams, which enable scanning of the sample spectrum with simultaneous substraction of the blank spectrum.
Technically Multiskan® Spectrum provides a zero to four absorbance unit (AU) read-out-range; 0-3 AU, ± 2% linearity; 0-2 AU, ± 0.005 AU accuracy; precision CV < 1%, 0-2 AU, CV < 2%, 2-3 AU precision; an optical bandpass of 2nm (± 1nm wavelength accuracy) and a stray light of <0.02% at 230nm.

To avoid non-specific absorbance from buffers or other chemical moieties, only Sterile-filtered deionised water was used. Prior to scanning, standard matched semi-micro cuvettes (0.5 ml) were shaken at (30rpm); scanning the absorbance spectrum was strictly carried out under the same conditions at 25°C at five nanometre wavelength interval first in the 200 to 100nm wavelength range. The absorbance maximum was confirmed by rescanning the samples in the ultraviolet region at one-nanometre wavelength interval. The absorbance at $\lambda_{\text{max}}$ (max = wavelength at which the maximum absorbance peak obtained) and absorbance at $\lambda_{\text{280nm}}$ were recorded. Samples with more than 0.005 AU variations were rejected.

5.3.6 Reversed-phase high-pressure liquid chromatography (RV-HPLC)

All chromatographic separations were performed on a Shimadzu LC (Class VP software) system, incorporating LC10Ai solvent delivery unit, online DGU-14A degasser, CT0-10A column oven, SPP-M10A Diode array detector, CDD-6A conductivity detector, and SCL-10A system control. A silica-based C$_{18}$ column, 5u, 250 X 4.6 mm (3354, Alltima, Alltech Associates, Inc.) was employed for separation employing an isocratic elution at solvent flow rate of 1.0 ml/min with HPLC-grade 100% acetonitrile (Labscan Asia Co., Ltd., Thailand) and 0.1% trifluoroacetic acid in double-deionised water (Pierce Chemical, USA).
Fifty microliter of eluant was filtered through 0.22μm filter and the effluent was monitored at 220nm. The column was washed with the mobile phase solvents until the absorbance at 220nm returned to the base line. High reproducibility of the retention time was obtained run to run. Due to the complexity in comparing the various chromatographs of tested milk protein solutions and the limited scope of the present study, chromatographs were used mainly for comparisons and verification of changes in the spectral profile of native, heat processed and modified milk BMPs.

5.4 Results

5.4.1 Fat and precipitant yield

Human breast milk and ISFS have almost a neutral pH (7.3 and 7.2, 25 °C, respectively). Slight acidity was identified in RWPBM, PHFBM, ISFN and ISFK, and adult formulae pH (mean ± standard deviation (STD) 6.7 ± 0.1 and 6.4 ± 0.1; 25 °C, respectively). The precipitants yield (percent wet weight) of HBM was the lowest among all analysed BMPs (0.15%) and the fat yield was relatively low (2.6%), whereas adult formulae have the highest precipitants yield (18.3% ± 0.01) and a trace of fat (0.04% ± 0.001). On the other hand, the fat and precipitants yield of ISF was 4.1% ± 0.01 and 1.7% ± 1.4*10^{-05}, respectively, and the fat yield of RWPBM and PHFBM was 5.5% and 6.2%, respectively and precipitants yield was 4.6% and 3.6%, respectively.
5.4.2 Identification standard curve

Human and BMPs have a relatively similar spectral profile in 250 to 310nm spectral region with variations in the intensity of the absorbance peak. A concentration dependent linear range identification standard curve was established for HBM and all tested BMPs. At a higher concentration range, the relationship was nonlinear. Figure 5-1 shows that while the concentration range required to establish a linear range for HBM was 100-fold higher than that required to BSA as well as BI (Fig 5-2). This was 10-fold higher than that required by RWPBM, PHFBM, adult and infant bovine-based canned formulae (Table 5-1).

Table 5-1 shows that while hypo allergic enzymatically hydrolysed whey protein infant formula (ISFN) yielded the highest absorbance maximum among all tested BMPs and HBM ($\lambda_{270}=1.902$ and $\lambda_{280}=1.758$) this was followed by adult skimmed formulae (Adult H and J), the absorbance maximum of whole pasteurised and homogenised bovine milk was the lowest ($\lambda_{275}=0.264$ and $\lambda_{280}=0.258$). It has been also demonstrated that while HBM and infant starter formulae have yielded its maximum absorbance at 270nm, adult and whole fresh and pasteurised bovine milk protein solutions have exhibited a maximum absorbance at $\lambda_{275nm}$. Distinctive chromatographic profiles of HBM and all tested BMPs are shown in figure 5-3.
Figure 5-1 Spectral profile of human breast milk (HBM). Milk solutions separated by precooling to 4°C/20min and centrifuged at 25,000 g/60min/4°C; 70mm diameter rotor. Absorbance obtained at λmax: 270nm (λ.max for wavelength at which the maximum absorbance obtained) and λ280nm.
Figure 5-2 Spectral analysis of native bovine serum albumin (BSA) and bovine insulin (BI). Absorbance obtained at $\lambda_{\text{max}}$: 275nm ($\lambda_{\text{max}}$ for wavelength at which the maximum absorbance obtained) and $\lambda_{280}$nm.
Table 5-1 Spectral of raw whole pooled bovine milk (RWPBM), pasteurised and homogenised fresh bovine milk (PHFBM), bovine-based adult canned formulae (Adult H, J and D), canned whey dominant bovine-based starter infant formulae (ISFS and ISFK), and canned enzymatically hydrolysed whey protein infant formula (ISFN). Milk solutions separated by precooling to 4ºC/20min and centrifuged at 25,000 g/60min/4ºC; 70mm diameter rotor. Absorbance obtained at $\lambda_{\text{max}}$: 270nm and 275nm ($\lambda_{\text{max}}$ for wavelength at which the maximum absorbance obtained) and 280nm.

<table>
<thead>
<tr>
<th>Milk product</th>
<th>Wavelength (nm)</th>
<th>Concentration range (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Fresh</td>
<td>RWPBM</td>
<td>$\lambda_{275}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\lambda_{280}$</td>
</tr>
<tr>
<td>Pasteurised</td>
<td>PHFBM</td>
<td>$\lambda_{275}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\lambda_{280}$</td>
</tr>
<tr>
<td>Bovine-based</td>
<td>Adult H</td>
<td>Skimmed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult J</td>
<td>Skimmed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adult D</td>
<td>Full cream</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine-based</td>
<td>ISFS</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>infant</td>
<td>ISFN</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>ISFK</td>
<td></td>
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</tbody>
</table>
Figure 5-3 Comparison between the chromatographic profiles of untreated human breast milk (HBM), raw whole pooled bovine milk (RWPBM), pasteurised and homogenised fresh bovine milk (PHFBM), adult full cream (Adult D), adult skim (Adult H) and infant starter formulae (ISF)K, ISFS and ISFN. Column (C$_{18}$) isocratic mobile phase solvents (100% acetonitrile and 0.1% trifluoroacetic acid in double-deionised water). Flow rate 1ml/min. Detection at 220nm.
5.5 Modification and heat processing

5.5.1 Modification of bovine serum albumin with bovine insulin

Concentration dependent changes in the spectral profile as well as the chromatographic profile of modified BSA with BI are shown in Table 5-2 and Figure 5-4, respectively. Unlike the incubation of equal volumes and concentrations of BSA (1mg/ml) modified with BI (1mg/ml) at room temperature (17°C/24h/25rpm, pH 7.4), the incubation of mBI-BSA under physiological conditions (37°C/24h/25rpm, pH 7.4) has yielded the highest absorbance peak, which was also higher than that of BSA modified with BI at physiological conditions with different alternative concentrations.
Table 5-2 The effect of incubation and concentration on the absorbance maximum of bovine insulin (BI, 1mg/ml), bovine serum albumin (BSA, 1mg/ml) and BSA modified with alternative different concentrations and equal volumes of BI (mg/ml) under physiological conditions (37ºC/24h/25rpm, pH 7.4) and under room temperature (RT: 17ºC/24h/25rpm, pH 7.4). Absorbance obtained at $\lambda_{max}$: 275nm (($\lambda_{max}$ for wavelength at which the maximum absorbance obtained) and at $\lambda_{280nm}$.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{275nm}$</th>
<th>$\lambda_{280nm}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin</td>
<td>0.73</td>
<td>0.74</td>
</tr>
<tr>
<td>Bovine insulin</td>
<td>1.05</td>
<td>0.94</td>
</tr>
<tr>
<td>BSA (0.2 mg/ml) / BI (0.8 mg/ml)</td>
<td>0.48</td>
<td>0.44</td>
</tr>
<tr>
<td>BSA (0.4 mg/ml) / BI (0.6 mg/ml)</td>
<td>0.46</td>
<td>0.43</td>
</tr>
<tr>
<td>BSA (0.6 mg/ml) / BI (0.4 mg/ml)</td>
<td>0.46</td>
<td>0.44</td>
</tr>
<tr>
<td>0.8BSA (0.8 mg/ml) / BI (0.2 mg/ml)</td>
<td>0.56</td>
<td>0.54</td>
</tr>
<tr>
<td>BSA (1.0 mg/ml) / BI (1.0 mg/ml)</td>
<td>0.95</td>
<td>0.89</td>
</tr>
<tr>
<td>PC: 37 ºC/24h/25rpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA (1.0 mg/ml) / BI (1.0 mg/ml)</td>
<td>0.56</td>
<td>0.56</td>
</tr>
<tr>
<td>(RT:17 ºC/24h/25rpm)</td>
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</tbody>
</table>
Figure 5-4 Chromatographic profile of bovine insulin (BI, 1mg/ml), bovine serum albumin (BSA, 1mg/ml) and BSA modified with alternative concentrations and equal volumes of BI (mg/ml) under physiological conditions (PC: 37°C/24h/25rpm, pH 7.4) and under room temperature (RT: 17 °C/24h/25rpm, pH 7.4). Column (C_{18}), isocratic mobile phase solvents (100% acetonitrile and 0.1% trifluoroacetic acid in double-deionised water). Flow rate 1ml/min. Detection at 220nm.
5.5.2 Heat processing of native and modified bovine serum albumin with bovine insulin

Compared to the unmodified and water bath heat processed BSA, changes in the absorbance maximum of mBI-BSA in reference to the not heat processed mBI-BSA were substantial. This was temperature and exposure time dependent (Figure 5-5 and 5-6). While BSA has exhibited obvious spectral changes at 50°C/5min, and 60°C/5min, the highest was at 70 °C/5min (36% at $\lambda_{275\text{nm}}$, and 33% at $\lambda_{280\text{nm}}$).

Modified BI-BSA has also shown substantial changes in the absorbance maximum at 40°C /10min, and 60°C /15min (97.7%, 48.4% at $\lambda_{275\text{nm}}$, and 100.3%, 49.3% at $\lambda_{280\text{nm}}$ respectively); the highest change was at 70°C /10min (119.5% at $\lambda_{275\text{nm}}$, and 122.3% at $\lambda_{280\text{nm}}$). Both BSA and mBI-BSA have shown a steady increase in their absorbance spectrum as subjected to higher temperatures (80, 90, and 100°C /5min). This was substantially higher in mBI-BSA absorbance spectrum activity with a slight milky discolouration.
Figure 5-5 Percent change in the effect of water bath heat processing at various temperatures and exposure time on the absorbance maximum of bovine serum albumin (BSA) and BSA modified with bovine insulin (mBI-BSA) under physiological conditions (37 °C/24h/25 rpm). The percent change in the absorbance maximum of heat processed BSA and mBI-BSA calculated in reference to that of untreated BSA and untreated mBI-BSA respectively. Absorbance determined at λ=280nm.
Figure 5-6 Effect of water bath heat processing at various temperatures and exposure time on the chromatographic profiles of bovine serum albumin (BSA), and BSA modified with bovine insulin (mBI-BSA) under physiological conditions (37 ºC/24h/25 rpm). Column C\textsubscript{18}, isocratic mobile phase solvents (100\% acetonitrile and 0.1\% trifluoroacetic acid in double-deionised water). Flow rate 1ml/min. Detection at 220nm.
5.5.3 Water bath versus hot plate heat treatment

To replicate home and industrial heat processes, milk proteins were heat processed by both water bath and hot plate heat processing. Figure 5-7 shows that all examined BMPs have shown considerable changes in their absorbance maximum as subjected to water bath heat processing with considerable variation among them. Infant starter formulae (ISFK and ISFS) have yielded the highest absorbance maximum, whereas enzymatically digested ISFN has shown a negative change in its absorbance.

While the highest positive percent change in the absorbance maximum of water bath heat processed BMPs was observed in ISFK, ISFN has exhibited the lowest percent change in its absorbance maximum with a negative trend throughout all temperatures applied (40-100°C/15min). Figure 5-9 also exemplifies that while only RWPBM has yielded a lower absorbance maximum as the water bath heat processing was replaced with hot plate heat processing procedure, ISFK and ISFS have yielded the highest change in their absorbance maximum as the processing method changed, whereas enzymatically digested (ISFN) formula has shown relatively lower change than that of ISFK and ISFS.
Figure 5-7 Effect of water bath heat processing (WB) at (40-100 °C for 15 min) and hot plate heat processing (HP) at (63 °C for 15 and 30 min) procedures on the percent change in the absorbance maximum of bovine serum albumin (BSA), raw whole pooled bovine milk (RWPBM), canned whey dominant bovine-based starter infant formulae (ISFS), and canned enzymatically hydrolysed whey protein infant formula (ISFN). The percent change in absorbance calculated in reference to that of not heat processed milk protein. Absorbance determined at $\lambda_{280\text{nm}}$. 

<table>
<thead>
<tr>
<th>Milk proteins</th>
<th>% Change absorbance at $\lambda_{280\text{nm}}$</th>
<th>WB 100 °C/min</th>
<th>WB 70 °C/min</th>
<th>HP 63 °C/15min</th>
<th>HP 63 °C/5min</th>
<th>WB 60 °C/15min</th>
<th>WB 40 °C/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>RWPBM</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ISFS</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ISFN</td>
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</tbody>
</table>
5.6 Discussion

Bovine milk is a very complex system with a minimum of 100,000 different molecular varieties. Many factors are capable of affecting the constituents of milk, for instance breed, cow to cow and breed to breed variations that encompass feeds, seasonal and geographic variations (Goff and Hill 1993). The appearance of both milk and milk products is influenced by their spectral characteristics that provides the basis for many rapid indirect methods of analysis, such as proximate analysis (Goff and Hill 1993; Goff 2004).

Infants experience rapid growth during their first year of life. Human breast milk is the ideal initial food for them (Wold and Adlerberth 2000; Oddy 2002). Mature and early human milk is heterogeneous (Viverge et al. 1990; Coppa et al. 1993; Newburg 2000) and its composition differs considerably from that of bovine milk and bovine milk-based infant formulae, which contain protein at a very low concentration as well as a high concentration of lactose (Goff and Hill 1993; National Health and Medical Research Council 2003).

Modern infant formulae require both large scale and home food processing that can bring about extreme alteration in the chemical composition of food products (Rechcigl 1982). Requirements to assimilate human milk high lactose content have made infants formula one of the most difficult-to-dry foods. Bovine milk is a complex polydispersion system and individual components of milk have various impact on the physical properties of milk (Pisecky 1997). The physical properties, viscosity, density and heat stability of milk are the most important properties to produce canned milk formulae (Pisecky 1997).
In spite of all efforts, it is not possible to duplicate the quality of human milk as well as the concentration of both macro and micronutrients content (Mayor 2004). Bovine milk contains more than 25 separate proteins, 80% of these proteins are heterogeneous casein occurring as a micellar complex. Whey proteins form the remaining 20% of the milk protein component (for example β-lactoglobulin (BLG), α-lactalbumin (ALA) and BSA) (Pisecky 1997; National Health and Medical Research Council 2003). Both adult and infant formulae require various processing procedures to meet ingredient and nutritional requirements.

Heat processing is one of the most frequently applied methods in milk-based foods processing technology. Thermal processing attributes to some undesirable possibly severe quality defects to bovine milk, a heat sensitive liquid, including the denaturation of the proteins (Pisecky 1997). Protein functional properties are completely dependent on its three dimensional structure (Kimball 2004). Heat treatment (Pisecky 1997), the presence or absence of co-denaturation associate, industrial thermal pre-treatment (Bertrand-Harb et al. 2002), and pH (Law and Leaver 2000) are all factors capable of disrupting protein structure.
Bovine milk proteins are widely implicated as an allergenic factors in infants (Goldman et al. 1963; Kletter et al. 1971b; May et al. 1982; Bahna and Gandhi 1983) as well as a potential triggering factors of the pancreatic autoimmune process (Karjalainen et al. 1992b; Saukkonen et al. 1994; Levy-Marchal et al. 1995a). It contains a minimum of 25 proteins capable of inducing immune responses (Bahna and Gandhi 1983). The antigenicity of certain milk proteins altered because of heat processing, BSA for example loses most of its antigenicity at 70 °C (Hanson and Mansson 1961; Strand 1994); it renders protection to casein (Pisecky 1997), a heat resistant milk protein (Hanson and Mansson 1961). Heat processed albumin go through reversible and irreversible stages of structural changes (Kuznetsov et al. 1975; Lin and Koenig 1976; Oakes 1976). Heat processing above 65ºC is regarded as an irreversible stage but does not necessarily result in a complete destruction of the ordered structure (Wetzel et al. 1980).

The pH of milk fluctuates within (6.5 to 6.7, 25 °C) with casein and phosphate acting as the main buffers (Goff and Hill 1993). Changes in the rate of heat denaturation with whey proteins at certain pH are complex and dependent on the rates of unfolding and aggregation phases (Law and Leaver 2000). Given that various heating processing methods affect the physical nature of milk proteins (Saperstein and Anderson 1962) as well as causing protein antigens to aggregate, (Hanson and Mansson 1961; Harlow and Lane 1988; Strand 1994; Alting et al. 1997), small changes in the structure of an antigen can influence intensely the vigour of the immune response (Harlow and Lane 1999). Unlike pasteurised whey protein or skimmed milk, antibodies produced against extremely heat processed whey protein were very low or undetected. If detected antibodies were residual casein specific that indicates an extensive denaturation of these proteins (Kilshaw et al. 1982; Heppell et al. 1984).
The modification and thermal treatment of various proteins can lead to the formation of new epitopes. This can alter the immune response to recognise the newly formed epitopes of the modified molecule rather than the native epitopes of the molecule. Bovine serum albumin is a thermo-labile milk protein and loses most of its antigenicity beyond critical temperatures 70 to 80ºC (Hanson and Mansson 1961; Karjalainen et al. 1992b; Savilahti et al. 1993; Boza et al. 1994; Alting et al. 1997). Modification of both BSA (Teale and Benjamin 1976) and bovine insulin (Speth and Lee 1984) with various binding molecule as well as heat processing have a substantial effect on their immunogenic response (Hanson and Mansson 1961; Karjalainen et al. 1992a; Alting et al. 1997). While the receptor activity of BSA is enhanced significantly if the modification was first of the amine terminal of arginine (Leibowitz and Soffer 1971), the modification of BSA with methoxypolyethylene has masked the antigenic sites of the modified BSA and was not able to induce any immunogenic response (Teale and Benjamin 1976). Modified bovine and porcine insulin have shown a high intramolecular mobility (Bak et al. 1967).

The findings of the present study are consistent with previous studies and provide further evidence that thermal processing of BMP can cause substantial changes in their spectral characteristics. Changes in the spectral profiles of milk proteins may be indicative of changes in the denaturation behaviour of milk proteins and indicative of the initiation of the aggregation process of heat processed milk proteins (Dalgleish 1990; de Frutos et al. 1992; Ferreira et al. 2001). Reports are helpful in representing the relative behaviour of different proteins; nonetheless data do not necessarily foretell the functionality of such proteins in real food systems which reflect the fact that in foods, substantial interactions with other ingredients may take place, resulting in modified behaviour of the proteins (Kinsella and Whitehead 1989).
5.7 Conclusion

The identification of bovine milk protein solutions by their characteristic spectral profile has provided further evidence on their heterogeneity. Both modification and thermal processing of bovine milk proteins have caused substantial changes in their spectral profiles that were concentration, temperature, and exposure times dependent. Whether these changes are indicative of changes in the immunogenicity of the modified and heat processed milk proteins require further investigation. It does seem encouraging to suggest that humanisation of the spectral profile of infant formulae may open a window to resolving controversy encircling the diabetogenicity of bovine milk proteins.
6 A newly developed saliva-based ELISA to determine immune response to milk proteins in young diabetic Jordanians: Lack of association for infantile feeding practices

6.1 Abstract

Objective: There is growing worldwide interest in the identification of potential environmental factors that may trigger the pancreatic autoimmune diabetes in genetically susceptible individuals. The possible immunogenicity of bovine serum albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) was examined.

Design and methods: Fifty diagnosed diabetic children under the age of 14 years and their unrelated age and gender matched control subjects were identified. Serum and saliva samples were collected and IgG and secretory IgA antibodies to BSA as well as to native and heat processed mBI-BSA were determined. Data on participants’ breastfeeding and early infant feeding practices were collected using constructed three-part questionnaire developed previously.
**Results:** Heated mBI-BSA (70°C/5min) has shown the highest relative change in the both IgG and sIgA titre levels, whereas mBI-BSA has also shown a substantial relative change in antibody titre levels compared to that of untreated BSA. Mean log effect of mBI-BSA on the titre levels of both IgG and sIgA antibodies in both DC and NDC has increased by approximately 1.3 fold of that of unheated or modified BSA (P = 0.000, and 0.114 respectively). The mean log effect of heat processed mBI-BSA at (70°C/5min) on IgG and sIgA titre levels in both groups has also increased by nearly 1.5 of that of native BSA (P = 0.000), and 1.1 fold of that of native mBI-BSA (P = 0.000). There were no immune responses to mBI-BSA heated at 70 °C/10min and 80°C/5min) and 60°C/5min). There were significant correlations between DC (60%) and NDC (25%) for the positivity of CRP (P = 0.05).

**Conclusion:** The saliva-based ELISA system may be a useful proxy of immune responses and may constitute new grounds for the ongoing dietary intervention trials. If bovine milk proteins are true diabetogens, the modification and thermal processing procedure of BSA is a merely sophisticated system in eliciting immune responses and is neither limited to diabetic patients nor associated with breastfeeding or early infant feeding practices, and may reflect unspecific defect of the immune system.
6.2 Introduction

None of the potential primary determinants, either genetic or environmental factors, of type 1 autoimmune diabetes has been established unequivocally (Kimpimaki et al. 2001; Akerblom et al. 2005; Kent et al. 2005; Nakayama et al. 2005). Nonetheless, a number of different complex mechanisms of pancreatic beta cell destruction are operative in type 1 autoimmune diabetes (Kawasaki et al. 2004).

Although bovine insulin is implicated as a putative primary autoantigen in animals (Atkinson et al. 1986; Srikanta et al. 1986; Komulainen et al. 1999; Kimpimaki et al. 2002; Nakayama et al. 2005), it is a small protein (hapten) (Harfenist and Craig 1952; Sanger 1958) and is unable to elicit immune response by itself. Rather it requires a linkage to a large immunogenic carrier, such as bovine serum albumin (BSA) (Carter and Ho 1994; Harlow and Lane 1999; Roitt et al. 2001).

The modification of either BSA (Teale and Benjamin 1976) or bovine insulin (Speth and Lee 1984) with different binding molecules as well as exposure to heat processing (Hanson and Mansson 1961; Karjalainen et al. 1992a; Strand 1994; Alting et al. 1997) can cause a substantial effect on their immunogenic response. Substantial interactions with other ingredients in food systems may result in the modified behaviour of the proteins (Kinsella and Whitehead 1989). Changes in the mucosal architecture as well as concomitant interactions between different dietary (Teale and Benjamin 1976; Speth and Lee 1984) and non-dietary constituents (Clarke 1975; Sharma et al. 1995b) coexisting in the gut environment can either boost or suppress the autoimmune process (Scott et al. 1996b).
The present study focuses on determining immune responses to modified and heat processed milk proteins particularly BSA and bovine insulin by using a newly developed saliva-based enzyme linked immunosorbent system (ELISA) in young Jordanian diabetic children. This will be with regard to their breastfeeding and early feeding practices of participants. In addition, this study may verify whether changes in the spectral profiles of BMP are indicative of alteration in their immunogenicity (Al-Domi et al. 2004).

### 6.3 Research design and methodology

#### 6.3.1 The population sample

The cohort of this retrospective case-control study consisted of 50 (36 females and 14 males) Jordanian diabetic children (DC) aged 14 years or less. Participants were identified by The National Centre for Diabetes, Endocrinology and Genetics, Jordan, and 50 (28 females and 22 males) unrelated, age and sex matched non-diabetic children (NDC) were identified by Princess Rahmah Children’s Hospital, and Al-Zahrawi Medical Laboratory, Jordan. Diagnosis of diabetes was confirmed by the medical history of the diabetic children, including the presence of clinical symptoms and the need for insulin therapy right after diagnosis. Participants were unrelated to those with diabetes or to each other. Control subjects were age and gender matched. Data on breastfeeding and early infant feeding practices were collected using a constructed questionnaire. Ethical approval was granted by the Human Research Ethics Committee of the University of Western Sydney as well as by the authorities of all Jordanian participating institutions. All subjects, or their parents when appropriate, gave either written informed consent or a witnessed verbal consent when appropriate.
6.3.2 Breastfeeding and early infant feeding practices

Data on breastfeeding and early infant feeding practices were modified from a previous study using a constructed questionnaire (Chapter 3, appendix 10.1 and appendix 10.2).

6.3.3 Blood samples collection

A licensed phlebotomist drew 10ml venous blood samples during routine consultation visits to the Hospital Outpatients Clinics by venipuncture into a clot (red top) test tube. Whole blood samples were stored overnight at 4°C and then centrifuged at 4°C/1000 rpm/15 min. Serum aliquots were stored frozen at -20°C (Jonstone and Thrope 1996). Two hemolyzed blood serum samples were discarded.

6.3.4 Saliva samples collection

Twenty saliva samples were collected from age-matched DC and their age and sex matched controls. Participants with periodontal diseases or who had undertaken major dental work and/or those prone to respiratory infections were excluded. To minimize the potential of saliva contamination particularly from dairy products, whole unstimulated samples were collected at least 30 minutes after the last meal or drink. Participants were asked to rinse their mouth thoroughly with water 10 minutes prior to sample collection.
Participants were encouraged to relax and visualize their favourite foods. Participants were seated comfortably (Miletic et al. 1996) and saliva samples (5-10 ml) were drawn by either passive slavering into a sterile swap tube or by a sterile plastic transfer suction pipette. Saliva samples were clarified by centrifugation at 4ºC/1000 rpm/15min (Castro et al. 2004). Aliquots were stored frozen at -20ºC. To break down mucopolysaccharides saliva samples were exposed to a single freeze-thaw cycle (Worthman et al. 1990; Shirtcliff et al. 2000).

6.3.5  **C - reactive protein (CRP)**

Blood serum of participants who had donated saliva samples were tested for total serum CRP by slide agglutination (CRP-Latex test) obtained from (Plasmatec Laboratory Products Ltd, UK). Specimens were tested for CRP positivity and negativity. The presence of agglutination indicates a level of CRP in the sample ($\geq$6 mg/l). Manufacturer’s specifications were maintained. Positive and negative controls were tested in parallel with each batch of tests.

6.3.6  **Preparation of the testing antigens**

Bovine serum albumin (BSA) and bovine insulin (BI) (Sigma-Aldrich MO, USA) were reconstituted in double deionised ultrafiltered water (0.18µm) and were filtered through 0.22µm filter (Millipore, UK). Bovine serum albumin (1mg/ml) was modified with an equal volume of BI (1mg/ml) under strictly aseptic physiological conditions (37ºC/24h/25rpm, pH 7.4).
Modified BSA with BI (m-BI-BSA) was subjected to water bath heat processing under the same conditions at 40ºC/10 min, 50ºC/5 min, 60ºC/5min, 60ºC/15min, 70ºC/5 min, 70ºC/10min and 80ºC/5min as described in chapter five (Al-Domi et al. 2004). Since this study was carried out in Jordan and to verify our developed spectrophotometric assay, the modification and heat processing of mBI-BSA were conducted strictly under the same condition (for more details see chapter 5) (Al-Domi et al. 2004).

6.3.7 Enzyme linked immunosorbent assay (ELISA)

A newly developed ELISA was used to determine serum IgG and sIgA tire levels. In brief, 96-well, high-binding flat-bottom microtitre plates (Greiner-Bio-One GmbH, Germany) were coated with 50 µl of (1 µg/ml) native BSA, native mBI-BSA, and mBI-BSA heat processed at different temperatures and various exposure times (40ºC/10 min, 50ºC/5 min, 60ºC/5min, 60ºC/15min, 70ºC/5 min, 70ºC/10min and 80ºC/5min) in (0.1M phosphate-buffered saline (PBS), pH 7.4, buffer 1) (Sigma-Aldrich, St Louis, USA). Plates were incubated for 12 hours at 4 ºC.

The coated plates were washed with 0.05% Tween 20 in buffer 1 (buffer 2) five times. The plates were blocked with 100µl (1%) normal sheep serum (DakoCytomation, Denmark) thermally treated at 50ºC/5min in buffer 2 and were incubated for one hour at 37 ºC then were washed with buffer 2 three times. Serum and saliva specimens were thawed and plates were coated with 50µl of both serum and saliva specimens. Two-fold dilution in buffer 1 was carried out for each of the specimens under the same conditions on the same plate this to avoid interassay variability. Coated plates were sealed and incubated for one hour at 37ºC followed by washing with buffer 2 three times.
Plates were coated with 50μl polyclonal alkaline-phosphatase-conjugated affinity purified rabbit anti-human IgG specific for Gamma-chains and alkaline-phosphatase-conjugated affinity purified rabbit anti-human IgA specific for Alpha-chains antisera (DakoCytomation, Denmark) diluted 1/3000 in buffer 2, incubated for one hour at 37°C and washed with buffer 2 four times. Liquid p-nitrophenylphosphate substrate (50μl) (Sigma-Aldrich, St Lois, MO) was added and the plates were incubated for six hours at 37°C. Reaction was stopped by adding 1 M NaOH and the end-point was measured at 405nm (ELX 800, Bio-Tek Instruments Inc.; KC junior software). Duplicates varying by more than 5% error were retested. All plates were sealed prior to incubation, and incubation was undertaken in a humidified incubator with a preset temperature.

Optical densities were subjected to point-to-point analysis. The cut-off point was determined as blank optical density [OD] *2. Serum and saliva titres were expressed as the 50% of the reciprocal dilution factor of OD just above the cut-off point. Titres less than or equal to the cut-off point were assumed zero.

6.3.8 Statistical analysis

All data were coded, computer entered and analysed using the Statistical Package for Social Sciences (SPSS). Assuming normally distributed errors, the data fit a linear regression model and the bivariate correlations procedure was used to compute Pearson’s correlation coefficient and Paired-Samples T-test, independent-Samples T-test and multivariate analysis of variance (ANOVA) parametric statistics were computed for serum and saliva logarithmic (log) titre levels.
Based on the values of a set of predictor variables, the logistic regression model was used to estimate odds ratios (OR) with 95% confidence interval (CI) for each of the independent variables in the model. Data were considered statistically significant at \( P<0.05 \).

6.4 Results

Figure 6-1 delineates relative change in the serum IgG and secretory IgA antibody titre levels produced to native bovine serum albumin (BSA), and native and heat processed modified BSA with bovine insulin (mBI-BSA) in both DC and NDC, with reference to that of BSA. While mBI-BSA has shown a substantial relative change in antibody titre levels, different heat treatment temperatures and exposure times have exhibited a wide range of changes in IgG and sIgA immune responses. Heat processed mBI-BSA (70ºC/5min) has shown the highest relative change in both IgG and sIgA antibody titre levels. Heat processing of mBI-BSA at 40ºC/10min, 60ºC/15min, 70ºC/10min and 80ºC/5min has shown a negative relative change in the IgG and sIgA antibody titre levels, whereas heat processing of mBI-BSA at 50ºC, 60 and 70ºC for five minutes has shown a positive trend.
Figure 6-1 Percent change (*100) in serum IgG and secretory IgA (sIgA) titre levels of diabetic children produced to modified and heat processed bovine serum albumin (BSA) with bovine insulin (mBI-BSA) at different temperatures and exposure durations (70°C/5min, 60°C/15min, 60°C/5min, 50°C/5min, 40°C/10min and untreated mBSA-BI). Relative change calculated in reference to that produced against untreated BSA. Percent change calculated in reference to IgG and sIgA antibody titre levels produced against untreated BSA.
Figure 6-2 compares logarithmic (log) mean effect that native bovine serum albumin (BSA) and native and heat processed mBI-BSA have on both serum IgG and secretory IgA antibody titre levels in diabetic and non-diabetic Jordanian children aged 14 years or less. The mean log effect of mBI-BSA on the antibody titre levels of both IgG and sIgA antibodies in DC and NDC has increased by approximately 1.3-fold of that of unheated or modified BSA (P = 0.000, and 0.114 respectively). The mean log effect heat processed mBI-BSA at 70ºC/5min has on IgG and sIgA antibody titre levels in both DC and NDC has also increased by approximately 1.5-fold of that of native BSA (P = 0.000) as well as 1.1-fold of that of native mBI-BSA (P = 0.000).

In addition, there was a significant difference for the increase in mean log serum IgG antibody titre levels produced to mBI-BSA heat processed at 50 and 60ºC/5min (P = 0.000) as well as to sIgA antibody titre levels produced against mBSA-BI heat processed at 50ºC/5min (P = 0.001). This was not significant to mBI-BSAS heat processed at 60ºC/5min (P = 0.155). Interestingly there were neither humoral nor secretory immune responses to mBI-BSA heated at 70 ºC/10min and 80ºC/5min. Furthermore, there were significant correlations between DC (60%) and NDC (25%) for the positivity of CRP (P = 0.05, Phi = 0.21), given the normal levels of ≥ 6 mg/l (Hayashi et al. 1970; Roitt et al. 2001).
Figure 6-2 Comparison between the sample paired test mean effect that native bovine serum albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) have on both logarithmic (log) serum IgG and secretory IgA (sIgA) antibody titre levels in both diabetic and non-diabetic Jordanian children aged 14 years or less. Mean effect was statistically significant at P<0.05 for all heat and modified BSA and BI.

* The effect of mBI-BSA (60°C/15 was statistically insignificant on sIgA antibody titre levels at P<0.05
Titre levels of IgG antibodies produced to native and heated mBI-BSA at 70ºC/5min were strongly associated with the diabetic and control group (r = -0.78 and -0.65, P = 0.000, respectively); IgG antibody titre levels to heat processed mBI-BSA at (50 and 60ºC/5min were also positively associated (r = -0.45, -0.43 and -0.32, P = 0.00, respectively). Similarly, sIgA antibody titre levels produced to heated mBI-BSA at 70ºC/5min were strongly associated with the diabetic and control group (r = -0.77, P = 0.000) that was not significant for native mBI-BSA.

Table 6-1 shows that there were statistically significant differences for tests of between-subjects effect in DC and NDC for interactions between IgG and sIgA antibody titre levels produced to unheated and not modified BSA, and native and heat processed mBI-BSA at different temperatures and exposure times. Modified BI-BSA has a strong negative association with log IgG antibody titre levels (r = -0.65, P = 0.000) that was (-0.25, P = 0114) for sIgA in both the diabetic and control group, whereas IgG and sIgA log antibody titre levels has the strongest association with mBI-BSA heat processed at 70ºC/5 min in both groups (r = -0.78 and -0.77, P = 0.000 respectively). The logistic regression analysis yielded that IgG and sIgA log antibody titre levels produced to untreated BSA, mBI-BSA and heat processed mBI-BSA in both DC and NDC was associated with a relatively similar risk of developing diabetes.
Table 6-1 Multivariate analysis of tests between-subjects; effects for native bovine serum albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) on serum IgG and secretory IgA (sIgA) titre levels in both diabetic and non-diabetic Jordanian children aged 14 years or less. Data were considered statistically significant at $P<0.05$.

<table>
<thead>
<tr>
<th>Between-antigens effects</th>
<th>F</th>
<th>P-Value</th>
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<tbody>
<tr>
<td>Anti-BSA sIgA and</td>
<td>5.211</td>
<td>0.029</td>
</tr>
<tr>
<td>anti mBI-BSA IgG</td>
<td></td>
<td></td>
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<tr>
<td>Anti-mBI-BSA sIgA and</td>
<td>4.836</td>
<td>0.035</td>
</tr>
<tr>
<td>anti-mBI-BSA IgG (60 ºC / 5 min)</td>
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<tr>
<td>Anti-mBI-BSA (50 ºC / 5 min) sIgA and</td>
<td>7.028</td>
<td>0.013</td>
</tr>
<tr>
<td>anti-mBI-BSA IgG (50 ºC / 5 min)</td>
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</tr>
<tr>
<td>Anti-mBI-BSA (60 ºC / 5 min) sIgA and</td>
<td>4.511</td>
<td>0.042</td>
</tr>
<tr>
<td>anti-mBI-BSA IgG (60 ºC / 15 min)</td>
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### 6.5 Discussion

To the best of the researcher’s knowledge, this study constitutes the first study on the immune response in young Jordanian DC aged 14 years or less with regard to their breastfeeding and early feeding practices. Worldwide, this study is also the first to develop a new saliva-based ELISA to determine the salivary IgA antibodies to dietary proteins particularly bovine milk proteins in diabetic children. This assay is non-invasive, rapid, accurate, valid and highly reproducible if proper standards and conditions are controlled to avoid unsystematic errors.
This includes number of factors. 1), age-matching, given that the levels of sIgA that follow the development and maturation of the salivary glands have a parabolic rapport with age (Smith et al. 1987; Ben-Aryeh et al. 1990; Kugler et al. 1992; Miletic et al. 1996; Percival et al. 1997); 2), saliva sample collection by means other than cotton (Dabbs 1991; Shirtcliff et al. 2001); 3), exclusion of patients with either periodontal diseases (Alaluusua 1983) or respiratory infections (Lehtonen et al. 1987; Atis et al. 2001); and 4), avoidance of contamination with dairy products (Magnano et al. 1989).

Saliva collection is a non-invasive, stress-free, simple method, and can be easily collected and stored (James-Ellison et al. 1997). Therefore, sIgA has been widely used as a biomarker in a number of fields. 1), biobehavioural studies (Ben-Aryeh et al. 1984; Worthman et al. 1990; Hertsgaard et al. 1992; Shirtcliff et al. 2000; Shirtcliff et al. 2001); 2), toxins and pollutants (Gilfrich et al. 1981; Bauer et al. 1983); 3), as diagnostic tool (Behets et al. 1991; Ciclitira and Ellis 1991; Kozlowski and Jackson 1992); and 4), to determine the levels of antibodies produced to gliadin in patients with celiac autoimmune disease (Ciclitira and Ellis 1991; Fasano et al. 2003).

Salivary IgA antibodies are one dominant characteristic humoral factor of the local immune system in the body secretions; therefore, it can act as first-defence line against local infections and as well as preventing the access of foreign antigenic factors to the immune system (Roitt et al. 2001). The high synthesis rate of the T cells dependent sIgA antibodies and the direct association with foreign factors as well as half-life and direct association with the activation of both T and B cells (Smith et al. 1987; Paul 1993) have made sIgA a better immune marker over measuring B and T cell in blood serum (Miletic et al. 1996).
Antibody immune responses at various mucosal effector sites are antigen type and dosage dependent. The whole mucosal immune system distant from the sensitisation site can be engaged by the sensitisation of a specific site of the system that may present a thorough view of the performance of the entire mucosal immune system (Jertborn et al. 1986; Externest et al. 2000). Changes in dietary ingredients, microbial flora (Sharma et al. 1995b; Sharma et al. 1995a), bacterial metabolites (Fontaine et al. 1996) and enteric viral infections (Hiltunen et al. 1997; Honeyman et al. 2000; Salminen et al. 2004) as well as interaction between these factors coexisting in the gut system may significantly change the mucosal architecture, the mucosal adaptation (Sharma and Schumacher 1995), the epithelial cell production (Clark et al. 1981), and the regional number of the enteroendocrine cells (Sharma and Schumacher 1996).

Therefore, stabilisation of the mucosal barrier may become an important factor important in gut health maintenance (Kleessen et al. 2003) that may affect gut permeability to possible foreign, dietary and non-dietary antigens and that impair secretory immunity. Substantial interactions with other ingredients in food systems may result in modifying the behaviour of proteins (Kinsella and Whitehead 1989).
The immunogenicity of antigens can often be profoundly altered by slight changes in the physical and chemical nature of the antigen; many molecules can be made more immunogenic by denaturation. Given that aggregate-free antigens may inhibit the immune responsiveness (Kletter et al. 1971b; Harlow and Lane 1988; Saukkonen et al. 1994), protein antigens aggregate caused by heat processing are usually more immunogenic. Heat treatment changes the structure of many compounds particularly proteins and expose new epitopes. This modification can either alter the regions of the immunogen to present better sites for T-cell binding or expose new epitopes for B-cell binding. While antibodies can differentiate between conformations of protein antigens, small structural changes of the epitope can prevent the recognition of the antigen. Antibodies can differentiate between conformations of protein antigens (Harlow and Lane 1988).

Bovine serum albumin particularly ABBOS peptide (Karjalainen et al. 1992a; Saukkonen et al. 1994), and insulin and proinsulin (B: 9-23 peptide) (Wegmann et al. 1994; Abiru et al. 2000; Alleva et al. 2000; Wong et al. 2002; Kent et al. 2005; Nakayama et al. 2005) are among the most putative environmental dietary proteins implicated in setting of the beta cell destruction. Insulin is one of the most strongly implicated as a potential primary autoantigen capable of initiating the destruction of insulin secreting beta cells sanctioning other pancreatic proteins to become the primary target leading to the disease. It has also been anticipated that insulin becomes a target ensuing the initiation of the autoimmune process by another autoantigen (Alleva et al. 2000; Juvenile Diabetes Research Foundation International 2005b; Nakayama et al. 2005).
Bovine insulin, differs from human insulin only at three amino acid residues (Yip et al. 1998), is a small hapten (5,808 Daltons) (Harfenist and Craig 1952; Sanger 1958) and cannot elicit an immune response by itself. For insulin to initiate an immune response, it requires coupling to a carrier protein such as BSA (66430.3 Daltons) (Harlow and Lane 1988; Carter and Ho 1994). The immunogenicity of antigens can often be profoundly altered by slight changes in the physical nature of the antigen; many molecules can be made more immunogenic by denaturation.

Given that aggregate-free antigens may inhibit the immune responsiveness (Kletter et al. 1971b), protein antigens aggregate caused by heat processing are usually more immunogenic. Heat treatment changes the structure of many compounds particularly proteins and expose new epitopes. This modification can either alter the regions of the immunogen to present better sites for T-cell binding or expose new epitopes for B-cell binding. While antibodies can differentiate between conformations of protein antigens, small structural changes of the epitope can prevent the recognition of the antigen. Antibodies can differentiate between conformations of protein antigens (Harlow and Lane 1988).
The modification and thermal processing of proteins can lead to the formation of new epitopes, which can alter the immune response to recognise the newly formed epitopes of the modified molecule rather than the native epitopes of the molecule. Modification of both BSA (Teale and Benjamin 1976) and bovine insulin (Speth and Lee 1984) with various binding molecule as well as heat processing have a substantial effect on their immunogenic response (Hanson and Mansson 1961; Karjalainen et al. 1992a; Alting et al. 1997). While the receptor activity of BSA is enhanced significantly if the modification was first of the amine terminal of arginine (Leibowitz and Soffer 1971), the modification of BSA with methoxypolyethylene has masked the antigenic sites of the modified BSA and was not able to induce any immunogenic response (Teale and Benjamin 1976). Modified bovine and porcine insulin have shown a high intramolecular mobility (Bak et al. 1967). Porcine insulin was capable of activating and binding to phosphatase to form a complex, whereas insulin chains as well as BSA were not capable of either the activation process or the binding process (Speth and Lee 1984).

Modern infant formulae require both large scale and home food processing, which can bring about extreme alteration in the chemical and physical nature of food products (Rechcigl 1982). Although the detection of antibodies associated with diabetes is technically demanding, antibodies to BSA are common in the general population (Levy-Marchal et al. 1995a). Bovine serum albumin has many epitopes capable of inducing a broad range of high and low affinity general antibodies population which can recognise both conformational epitopes as well as changes due to the denaturation of BSA (Karjalainen et al. 1992b; Savilahti et al. 1993; Saukkonen et al. 1994).
Bovine serum albumin is a thermo-labile milk protein and loses most of its antigenicity beyond critical temperatures 70 to 80°C (Hanson and Mansson 1961; Karjalainen et al. 1992b; Savilahti et al. 1993; Boza et al. 1994; Alting et al. 1997). Thermal processing of canned infant formulae as well as liquid formulae at different temperatures and various exposure times has rendered milk free of BSA (Hanson and Mansson 1961; Monte et al. 1994; Strand 1994; Alting et al. 1997). Patients sensitive to BSA might tolerate BMP subjected to heat treatment, and to some extent, they are less tolerated by those who are responsive to other protein fractions (Crawford 1960). Animal studies (Kilshaw et al. 1982; Heppell et al. 1984) have shown that unlike pasteurised whey proteins or skimmed milk, antibodies produced to extremely heat treated whey proteins (100 or 115°C for 30 minutes) were very low or undetected; if detected antibodies were residual casein specific that indicates an extensive denaturation of these proteins.

Studies on the thermo lability of insulin and its role in the diabetogenicity of insulin remain scarce. Nonetheless, its widely accepted that for insulin to maintain its viability, insulin-dependent diabetic patients are encouraged not to stored insulin vials under extreme temperatures (<2 or >30°C) (American Diabetes Association 2002). Furthermore, insulin suspensions exposed to temperatures more than 25°C for extend periods may become difficult to homogenise, whereas espousing insulin to 50°C or more leads to the coagulation of the insulin suspensions (Brange 1987) studies on the thermo lability of insulin and its role in altering the immunogenicity of the insulin molecule remain scarce. It is, therefore, pivotal to examine the effect of both modification and thermal processing on the diabetogenicity of insulin, given that canned infant milk formulae undergo a wide range of both large and home scale heat processing (Pisecky 1997).
Home scale preparation methods of infants liquid bottle milk feeds vary between the application of severe heat processing temperature and extended exposure times (boiling for an extended exposure times) to a less severe heat processing temperature and shorter exposure times, for example the application of microwave fields (Singh et al. 1998). Recent indications on the possibility to manipulate the initiation of pancreatic autoimmune process by dietary intervention in infancy implies that different modification as well as processing procedures of bovine milk proteins (conventional bovine-based infant formula versus casein hydrolysate-based infant formula) may affect its immunogenicity (Akerblom et al. 2005). Therefore, controlling the variability in protein composition and protein modification as well as the degree of protein denaturation requires further processing procedures appropriation and investigation.

Numerous physiological consequences are caused by adverse reactions to food that can be either immunologically or non-immunologically mediated reactions resulting in a wide range of signs and symptoms (Miller 1998). The majority of people with high circulating levels of antibodies produced against a number of common microorganisms often with highly immunogenic antigens (Miller 1998) such as viruses which among a wide range of microorganisms can be also found in foodstuffs including milk products (Vela 1997; Miller 1998). While circulating antibodies to food proteins are also common in the general population (Kletter et al. 1971a), antibodies to bovine milk proteins are weaker and less common in adults than in children (Kletter et al. 1971a). The development of a state of unresponsiveness to dietary proteins such as bovine serum albumin in adults can be a result of prolonged minimal antigenic stimulation (Korenblat et al. 1968).
Antibodies to bovine milk protein in neonates are often very low or absent, if present it may originate from the maternal circulation (Kletter et al. 1971a). While children born to diabetic mothers have maternally acquired antibodies that can continue to at least nine months after birth, increased islet autoantibody levels in infants is most likely a sign of de novo production of autoantibodies associated with T1ADM (Ziegler et al. 1999). The production of the antibodies in neonates can be initiated within eight days of exposure reaching the highest levels at three to fifteen months of age where the production of antibodies start to decrease with age (Kletter et al. 1971a).

On the surface it would appear to be a logical association with the risk or protection from diabetes, nevertheless evidence on the use of breastfeeding or early infant feeding restrictions to prevent or minimise the disease occurrence is of limited strength and should not be misinterpreted by parents and the public (Atkinson and Gale 2003). Human retrospective studies remain discrepant and controversial, and are featured by a number of factors. First, differences between low and high risk populations examined (Esfarjani et al. 2001). Second, the actual exposure to certain dietary and non-dietary diabetogenic factors (Norris et al. 2003). Third, the timing of exposure to triggering factors (Kimpimaki et al. 2001). Fourth, ethical implications particularly in preventive trials (Rosenbloom et al. 2000). Finally, the design and analysis methods used in studies (Karjalainen et al. 1992b).
The determination of the circulating antibodies to bovine milk proteins by ELISA remains a convenient and rapid method. Nevertheless, given that the findings of the studies on the diabetogenicity of dietary proteins remain discrepant and that the valence of certain proteins, for instance BSA in solutions is 5-fold higher than that on solid phase immunoassays (Harlow and Lane 1988; Pesce and Michael 1992), a number of problems can be identified with regard to the validity and reliability of the method (Miller 1998). These include; 1), variation between assays methods (Rowe et al. 1972; Matthews et al. 1980; Karjalainen et al. 1992b); 2), limitations of steric and diffusion activity (Pesce and Michael 1992); 3), the lack of an accepted golden standard to measure against; 4), binding to solid phase may denature the native epitopes that may cause unintended formation of different type of binding characteristics; 5), antigen absorption and desorption from the surface is a time and surface-dependent, which affects the quantity and stability of a bound protein; and 6) the absence of a reliable and valid method to determine the number of non-specific antigen-antibody interactions (Miller 1998). Therefore, findings on the immune responses to non-genetic determinants of type 1 autoimmune diabetes require cautious interpretation.

The findings of the present study indicates that immune responses were neither limited to diabetic patients nor associated with the breastfeeding or early infant feeding practices. A higher proportion of diabetic children received no bottle-feeding in their early infancy reflecting an unspecific defect of the immune system in young Jordanian children. Variation between low and high-risk populations requires further investigation.

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6.6 Conclusion

The newly developed saliva-based ELISA is non-invasive, rapid, and convenient method and constitutes a useful proxy of immune response studies in young diabetic children. Heat processing of proteins at various temperatures and exposure times cause substantial change in the secretory and humoral immune responses with significant variations. This verifies that changes in the spectral profiles of proteins are useful indicators for altered immunogenicity of proteins caused by physical and chemical treatment (Al-Domi et al. 2004).
7 Future prospects

Type 1 autoimmune diabetes is a lifelong degenerative disease. Even with good health care systems, the risk of developing long-term retinopathy, neuropathy, and nephropathy complications is increased. Given the knowledge that the prevention of the disease is not yet possible, minimising or delaying the destruction of the beta cell remains of great benefits particularly to individuals at high risk. Achieving ample insights into the immunopathogenesis of the disease, and the identification of individuals for prevention trials may be directed to a more efficient early diagnosis prior to the manifestation of the disease. Success in the ongoing research on preventing the disease in animals and the exciting findings about its causes are imperative for diagnosis, treatment, and minimising or even preventing the onset of the disease in humans.

The prevention of any disease requires identification of individuals at risk, identification of the causative factors and understanding of the mechanisms of the aetiopathogenesis of the disease (Becker et al. 2000). The aetiopathogenesis of type 1 autoimmune diabetes remains unknown. Nonetheless, a complex interplay between a number of mechanisms of beta cell destruction are operative in the disease (Kawasaki et al. 2004). This requires both genetic and non-genetic determinates for potentially harmful events to direct the autoimmune process (Ellis and Atkinson 1996; Akerblom et al. 1997; Visvanathan and Zabriskie 2000; Rich and Concannon 2002). Hitherto, none of these determinants that may either boost or weaken the immune response has been identified unequivocally (Kimpimaki et al. 2001; Akerblom et al. 2005; Kent et al. 2005; Nakayama et al. 2005).
The findings of the present study have demonstrated that the modification of dietary proteins particularly bovine milk proteins has caused substantial variations in the immune responses of both diabetic and non-diabetic children. Similarly, heat processing of the modified proteins at different temperature and exposure times has significantly altered the immune responses with wide variations. These findings are consistent with the knowledge indicating that substantial interactions with other ingredients in the food systems can result in modifying the behaviour of the proteins (Kinsella and Whitehead 1989). Even slight changes in antigen structure can profoundly affect the immunogenic interactions (Harlow and Lane 1999).

The present study was also able to develop two simple tools that can constitute new grounds for future studies on the modified immunogenic behaviour of dietary diabetogens in human trials. The first tool was used to identify changes in spectral profiles of modified and heat processed milk proteins, and the second tool was a new, non-invasive, rapid and convenient saliva-based enzyme linked immunosorbent assay to determine the secretory immune response to these proteins retrospectively. It is pivotal to examine the effect of both large scale and home heat processing procedures as well as the modification of various milk proteins on the diabetogenicity of milk proteins in suitable animals models and consequently in longitudinal human studies. This can play a crucial role in our understanding of the disease aetiopathogenesis.
If dietary proteins particularly milk proteins are true diabetogens (Harrison and Honeyman 1999; Wegmann and Eisenbarth 2000; Nakayama et al. 2005), bovine insulin and bovine serum albumin remain among the most putative non-genetic dietary diabetogenic factors implicated in the initiation of beta cell destruction. Nonetheless, given the evidence on the possibility of manipulating pancreatic beta cells autoimmunity by dietary interventions in infancy by using infant formulae subjected to complex heat and enzymatic processing procedures (Akerblom et al. 2005), investigation of the nature and effect of receptor behaviour of the coexisting milk proteins in their natural milk system, the nature and effect of both large and home scale heat processing procedure on the diabetogenicity of dietary proteins should provide novel insights into the detrimental role these factors may have in the aetiopathogenesis of the disease in humans.

A number of factors can influence the development of type 1 autoimmune diabetes. These factors include: (1) variations in amount (Westrom et al. 1985; Scott 1990; Dahl-Jorgensen et al. 1991; Gerstein 1994) and type of milk proteins consumed (Thorsdottir et al. 2000), (2) cleanliness of the environment (Harrison and Honeyman 1999), (3) mucosal mechanisms and exposure to various dietary constituents, (4) substantial interactions with other ingredients in food systems which can result in a modified behaviour of proteins (Kinsella and Whitehead 1989), (5) changes in the mucosal architecture, and (6) interaction between different dietary (Teale and Benjamin 1976; Speth and Lee 1984) and non-dietary constituents (Clarke 1975; Sharma et al. 1995b) coexisting in the gut environment. All can influence both the immunogenicity of these factors and the maturation of the gut mucosa and therefore the macromolecular uptake of possible foreign diabetogens (Sharma et al. 1995b; Sharma et al. 1995a; Fontaine et al. 1996; Hiltunen et al. 1997; Honeyman et al. 2000; Westerholm-Ormio et al. 2003; Salminen et al. 2004). This process can either boost or suppress the autoimmune process (Scott et al. 1996b) and can play, among other factors and mechanisms, a critical role in the destruction of pancreatic beta cell (Harrison and Honeyman 1999; Kawasaki et al. 2004).
Although on the surface it would appear to be a logical association with the risk or protection from diabetes, evidence on the use of breastfeeding or early infant feeding restrictions to prevent or minimise the disease occurrence is of limited strength and should not be misinterpreted by parents and the public (Atkinson and Gale 2003). Emerging findings on gene therapy (Zalzman et al. 2003; Sapir et al. 2005) and cellular immunotherapy (Ogasawara et al. 2004; Tang et al. 2004; Tarbell et al. 2004) as well as studies on the regeneration of insulin-producing pancreatic beta cells may be harnessed as novel therapeutic targets for type 1 autoimmune diabetes (Juvenile Diabetes Research Foundation International 2004). Eventually, if the genetic and non-genetic determinants of the disease are established unequivocally; the benefits to individuals and the world society will be enormous. Therefore, studies embarking on identifying the nature and timing of the primary environmental factors that may predispose to the initiation of the disease should continue (Kin et al. 2005; Mirenda et al. 2005). Mind map III exemplifies the aspects of future studies.
7.1 Mind Map III: Aspects of future studies
8 Conclusion

The present study has addressed the heterogeneity of milk proteins and the effect modification and heat processing have on altering the immunogenicity of dietary proteins particularly bovine serum albumin and bovine insulin in Jordanian diabetic children aged 14 years or less with regard to their breastfeeding and early infant feeding practices and has drawn the following conclusions:

- Global geographic variation in the disease occurrence among children aged 14 years or less is significantly associated with worldwide variations in both mean and relative change in both total milk imports excluding butter and the amount of milk proteins consumption and is influence by the socioeconomic status of populations. Whether these variations are indicative of variation in the diabetogenicity of bovine milk proteins requires further longitudinal studies.

- Breastfeeding in Jordan is common and is usually extended for a minimum of one year for the majority of children. No significant associations were established for neither breastfeeding nor early infant feeding practices between both DC and NDC Jordanian children aged 14 years or less. A higher proportion of diabetic children received no bottle-feeding in their infancy. The majority of children were diagnosed with diabetes in winter at approximately six years of age and were female predominant.
• Dietary compliance in diabetic children was poor with signs of undernutrition in both groups as well as signs of obesity among NDC. For better nutrition compliance, there does seem a need for better dietary monitoring and professional dietary counselling programmes for both diabetic and non-diabetic children as well as establishing a national diabetes registry and developing national dietary guidelines.

• Blood group O+ is significantly predominant in young Jordanian children with diabetes and is associated with a greater risk of developing diabetes. A higher proportion of NDC has Rh (D) negative blood groups with gender variation. Whether certain blood groups confer a protective effect from diabetes requires further investigations on the secretory status of humans with type 1 autoimmune diabetes.

• The identification of bovine milk protein solutions by their characteristic spectral profile has ascertained their heterogeneity. Both modification and thermal processing of bovine milk proteins have caused substantial changes in their spectral profiles. These changes were concentration, temperature, and exposure times dependent. Changes in the spectral profile of proteins are indicative of changes in the immunogenicity of processed proteins. Humanisation of the spectral profile of infant formulae may open a window to resolving controversy encircling the diabetogenicity of bovine milk proteins.

• Newly developed saliva-based ELISA is non-invasive, rapid, and convenient method and constitutes a useful proxy of immune response studies in young diabetic children.
• The modification as well as heat processing of bovine serum albumin with bovine insulin (mBSA-BI) at various critical heating temperatures and exposure times has triggered different secretory and humoral immune responses with substantial variation. Various temperatures and exposure times caused different immune responses. While secretory and humoral immune responses to mBSA-BI heat processed at 70°C/5 min has enhanced substantially, heat processing at 70°C/10min and 80°C/5min has masked the immune responses. These findings verify that changes in the spectral profiles of proteins are useful indicators for altered immunogenicity of proteins caused by physical and chemical treatment.

• The immune response in young Jordanian children is neither limited to diabetic patients nor associated with the breastfeeding or early infant feeding practices. A higher proportion of diabetic children received no bottle-feeding in their early infancy reflecting an unspecific defect of the immune system. Variations between low and high-risk populations require further investigation.

The concomitant interactions of the coexisting dietary and non-dietary factors, interpopulation, and individual variations seem to be a more complex process than previously anticipated. Changes in the mucosal architecture as well as interactions between different dietary (native, modified, and thermally processed), and non-dietary (viral, normal flora) factors coexisting in the gut environment may either boost or suppress the pancreatic autoimmune process. Therefore, the examination of these factors, which may intervene concomitantly in a given individual(s) or in a population(s) as independent factors per se, may constitute a major source of error in the understanding and interpretation of the aetiopathogenesis of the pancreatic autoimmune process. Changes in these factors would need a longer period of time to change and would occur concomitantly in different individuals as well as in different populations, therefore extended longitudinal studies are necessary.
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mediated autoimmunity: viral peptides activate human T cell clones specific for


10 Appendices
10.1 Appendix A: Information sheet, consent, and survey (English)

10.1.1 Participant information sheet (English)

Dietary proteins & the development childhood type 1 (insulin-dependent) diabetes mellitus among newly diagnosed Jordanian children

Principal researchers: Dr. Mark R. JONES and Dr. Jim BERGAN

Investigator: Hayder AL-DOMI

Investigator’s contact details: School of Science, Food & Horticulture, University of Western Sydney, Australia, Richmond Campus, K-12, Locked Bag 1797 Penrith DC NSW 1797, Australia. Telephone: 0796498736 (Mobile / Jordan). E-mail: h.aldomi@uws.edu.au

Note: This research has been approved by the University of Western Sydney Human Ethics Committee (HEC 02/162). If you have any complaints or reservations about the ethical conduct of this research, you may contact the Human Research Ethics Committee through the Research Ethics officers (telephone: 0061-2 4570 1136). Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Questions and inquiries: Please if you have any questions or inquiries do not hesitate to contact the researcher at any time convenient for you.

THANK YOU
Introduction

Infancy growth

In any culture, for each infant, child, or adolescent, food nurtures both the physical and the personal “grow up” process. Food, feeding, and eating during these highly significant years of childhood do not and cannot exist apart from the broader overall process of growth and development. The whole process produces the whole person. Infancy growth is rapid during the first year of life. Most infants double their weight by the time they are 6 months of age and triple it by the age of 1 year. The ideal first food for the infant is human milk. It provides essential nutrients in quantities uniquely suited for optimal infant growth and development. Positive growth and development of healthy children depend on optimal nutritional support. From birth, as children grow older, their nutritional needs change with each unique growth period. Infants experience rapid growth. Human milk is preferred as their first food, with solid foods delayed until about 6 months of age.

Breastfeeding

Breastfeeding can be successfully started and maintained by most mothers who try, have support, and have an increased diet for lactation. Breasts are prepared for lactation during pregnancy. As the infant grows, the breast milk develops and adapts in composition to match the needs of the developing child.

Advantages of breastfeeding

There are many physiological and practical advantages to breastfeeding, including: (1) fewer infections, (2) fewer allergies, (3) ease of digestion and (4) convenience and economy. If the mother does not choose breastfeeding, or some condition in either mother or baby prevents it, bottle-feeding of an appropriate formula, usually cow’s milk-based formula is an accepted alternative.
Diabetes mellitus

Diabetes is a disease characterized by high blood glucose levels, resulting from either insufficient or no insulin release by the pancreas. Type 1 (insulin-dependent) diabetes is a form of diabetes in which the person with the disease requires insulin therapy. Type 1 diabetes often begins in late childhood, but can occur at any age. It runs in certain families, indicating a genetic link.

Most cases of type 1 diabetes begin with an immunological disorder. Our own immune system fails to distinguish between its own body and foreign agents leading to variety of autoimmune disease, for example rheumatoid arthritis, rheumatoid fever, multiple sclerosis and type 1 diabetes. In type 1 diabetes, the autoimmune disorder causes destruction of insulin producing cell in the person’s pancreas.

This disorder is, most likely triggered by a foreign virus or protein foreign to our bodies setting off the destruction. In response to pancreatic cell destruction, eventually the pancreas loses its ability to synthesize insulin and the clinical stage of the disease begins. Cow’s milk-based infant formula is the first foreign proteins introduced into our children’s diet. Thus, it has undergone extensive and intensive investigations. Nonetheless, no causal links between introduction of foreign proteins and the development of the disease have yet been established unequivocally, there is no scientific evidence to support this hypothesis.

Magnitude of Diabetes among Jordanian children

Despite the great advances in control and treatment, diabetes mellitus remains a major cause of morbidity and mortality throughout the world. Recent estimates suggest that more than 100,000 inhabitants in the Middle East suffer from type 1 insulin-dependent diabetes and that about 6000 subjects in the region develop the disease each year, and of these some 3000 are children below the age of 15 years. In 1997, there were around 5200 Jordanians experiencing the disease. Of them, some 700 are children below 15 years old. The occurrence of type 1 insulin-dependent diabetes mellitus in Jordanian children aged 0-14 years is among the lowest in the region, but is increasing. Data indicate that between 1992 and 1996 the occurrence of type 1 insulin-dependent diabetes has increased from 2.8 per 100,000 children in 1992 to 3.6 per 100,000 children in 1996.
Aim and benefits of the study

The increasing occurrence of the disease among Jordanian children has encouraged us to study whether the introduction of certain dietary proteins at a particular age has any effect on the initiation of insulin producing pancreatic cells or no. Thus, the main aim of this study was to identify the changes that persevere up to the initiation of insulin-producing pancreatic cells autoimmunity process, and to study the possible role cows’ milk proteins may have in provoking the pancreatic autoimmunity process in Jordanian young children. That is in order not only to minimizing or stopping progression of the ongoing process, but also to prevent the initiation of beta-cell destruction.

Objectives of the study

The main objectives of the study are:

- to establish a general dietary profile for young diabetic Jordanian children compared with non-diabetic children, and to study possible changes in feeding practices if the child develops the disease; and

- to study the possible role cows’ milk proteins may have in the development of the disease by determination of saliva and/or blood serum antibody levels may produced against certain cows’ milk proteins.

Importance of your participation

The rational of the study hinged upon improving our understanding of the disease. You are participating in this research to help scientists understand the reasons behind the increasing level of the diabetes in children throughout the world and in Jordan as well. Our understanding will help in setting new preventive measures to control or minimise the occurrence of the disease.
Your part in the study

- Your permission is required for your child to participate in this study. You will be asked to sign a consent form.

- Filling in a questionnaire that will help us to establish a dietary profile for your child, and to study your feeding practices if the child develops the disease.

- Donating a saliva sample. That will enable us to determine antibodies, which might be produced against certain cows’ milk proteins. Please consider not feeding the child at least 30 minutes before giving the saliva sample, and rinsing the child’s mouth with water just before the collection.

- To compare the levels of antibodies that may be present in the child’s saliva with that in the child’s blood; upon your agreement, a licensed blood drawer will collect a blood sample. Primarily only one saliva and/or blood sample will be collected. The total process will take approximately 20 minutes.

Harms and risks in participation

There will be little risk or harm in participating in this study. A licensed blood drawer will obtain the blood sample; nonetheless, you may notice a slight redness at the site of the needle prick. The procedure will be carried in a clean room, and only one-use needles will be used. The site of needle prick will be cleaned properly before and after the needle insertion. A false needle prick is not expected, but if it does happen, a repeat blood drawing will take place ONLY with your approval. Psychologically, the infant may encounter uneasiness and fear; therefore, parents will be asked to and involved in relaxing, reassuring and calming the infant. The blood drawer will take every professional effort to draw the sample swiftly. The room will be as comfortable as possible. It is unforeseen that collecting saliva samples will result in any stress/distress. Nonetheless, the participant may feel discomfort. Thus, we will make sure that the participant is relaxed before swabbing the sample with his/her parent(s) assistance.
Privacy & confidentiality

We will maintain the highest degree of confidentiality, personal, and information privacy. No individuals will be identified at any stage during the research or any future publication of the information. All documents related to the informed consent will be stored in a safe place separately from the data, which will be kept securely in a safe place for the mandatory years then destroyed. You are completely free to stop participating in this study at any time or to withdraw prematurely. In addition, there are no disadvantages/penalties or adverse consequences for not participating or for stopping participation in this research.

New findings

If new and conclusive information becomes known during the research, you will be informed if you have requested such information. The results of the research will be published in relevant scientific journals. Nonetheless we are pleased upon you request to supply you with a results summary report.

This research summary is your copy and you may keep along with a copy of the Consent Form
10.1.2 Consent to participate in research (English)

**Title of Research Project:** The Relationship Between Dietary Proteins and the Development of Type 1 (Insulin-Dependent) Diabetes Mellitus in Young Jordanian Children by Examining Susceptible Blood Groups and the Levels of IgA and IgG

Name of Researcher: Hayder AL-DOMI

- I understand that Mr. Al-Domi is a PhD student studying the effect dietary proteins might have on the development of insulin-dependent diabetes mellitus among Jordanian children. The researcher will conduct this study in a manner conforming with ethical and scientific principles set out by the University of Western Sydney-Human Research Ethics Committee (UWSHREC), Australia.

- I acknowledge that I have read, or have read to me the Participant Information Sheet relating this study. I acknowledge that I understand the Participant Information Sheet. I acknowledge that the general purpose, methods, demands and possible risks and inconveniences that may occur to me during the study have been explained to me by __________________________ (“the researcher”).

- I understand that since my child is under the age of 14 years, I have to sign on behalf of my child.

- I acknowledge that I have been given time to consider the information and to seek other advice.

- I realize that the study will take approximately 20 minutes of my time, and will involve filling in a questionnaire.

- I understand the general purposes, methods, demands and possible risks and inconveniences that may occur during the study.

- I understand that participation in the study might cause some physical and/or psychological stress to my child.

- I acknowledge that my participation in this study is strictly voluntary, and I may withdraw at any time.
• I know that I have the right to withdraw at any time and that the care of my family member and my relationship with the health care team will not be affected.

• I acknowledge that this research has been approved by the UWSHREC.

• I acknowledge that I have received a copy of this form and the Participant Information Sheet.

• If I have any question about the study or about being a subject, I know I can call Mr. Al-Domi. I may reach him at (++961-2-45703216 [Work], ++9611-2-968 75816 [home] and ++961-405543209 [mobile], Australia, and at (02-7046118 [home] and 0796498736 [mobile] Jordan.

• I agree to participate in this study, and I have been assured that the highest degree of confidentiality, personal and information privacy will be observed. My identity and my child’s identity will not be revealed while the study is being conducted or when the study is published.

• I am informed clearly that all documents related to the survey and the signed informed consent will be stored in a safe and confidential place separately from the data for the university’s five mandatory years; then documents will be destroyed according to the university’s policy.

Date:

Name of Participant: _________________________ Date of Birth: _________________________

Address of Participant: __________________________________________________________

Signature of Participant (if applicable): __________ Date: ___

Name of Participant’s Parent: _____________________________________________________

Address of Participant’s Parent (if different)

Relationship to participant:
10.1.3 Survey form (English)

**Title of Research Project:** The Relationship between Dietary Proteins and the Development of Type 1 (Insulin-Dependent) Diabetes Mellitus in Young Jordanian Children by Examining Susceptible Blood Groups and the Levels of IgA and IgG.

Dear Sir /Madam,

I am Hayder Al-Domi, a PhD student at University of Western Sydney studying the possible effect dietary proteins have on the development of type 1 (insulin-dependent) diabetes mellitus among Jordanian children.

This study has been approved by the University of Western Sydney Human Research Ethics Committee (HEC 02/162). If you have any complaints or reservations about the ethical conduct of this research, you may contact the ethics Committee through the Research Ethics Officers (phone: 0061-2-4570 1136). Any issues you raise will be treated in confidence and investigated fully, and you will be informed of the outcome.

Please find attached a research summary (participant Information Sheet). It is your copy and you may keep it along with a copy of the Consent Form. The attached survey form consists of six parts, please tick, or fill in as appropriate.

If you have any questions, or you want to know more about the study and its outcomes, please do not hesitate to contact the researcher using the following contact details.

**Researcher’s Contact details:**

**Telephones:**
0796498736 (Mobile/Jordan),
0061-2-96875816 (Home/Sydney), and
0061-405543209 (Mobile/Sydney)
**E-mail:** h.aldomi@uws.edu.au
**Mailing address:** Richmond Campus, K-12,
Locked Bag 1797, Penrith DC NSW 1797

Thank you for participation
Part 1: General

Child’s name (optional): ---------------------------------------------
Mother’s name (optional): --------------------------------------------
Address (optional): -------------------------------------------------
Telephone (optional): -----------------------------------------------

Please tick or fill in as appropriate

1. Who is completing this survey?
☐ The mother of the participant.
☐ The father of the participant.

2. Do you have a child with diabetes?
☐ Yes
☐ No, please go to question 4.

3. If YES to question (2), do you agree to:
Filling in a questionnaire. ☐ Yes ☐ No
Allow swapping a saliva sample. ☐ Yes ☐ No
And/or drawing a blood sample. ☐ Yes ☐ No

4. If NO to question (2), do you like to include your child as a control?
☐ Yes
☐ No

If YES to question (4), do you agree to:
Filling in a questionnaire. ☐ Yes ☐ No
Allow swapping a saliva sample. ☐ Yes ☐ No
And/or drawing a blood sample. ☐ Yes ☐ No
### Part 2: Participant Demographic Data

<table>
<thead>
<tr>
<th>Age (Months)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Birth weight (kg)</th>
<th>Birth order</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Participant Blood group

- [ ] I do not know
- [ ] A+
- [ ] A−
- [ ] B+
- [ ] B−
- [ ] AB+
- [ ] AB−
- [ ] O+
- [ ] O−

### Part 3: Parents Demographic Data

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Weight (Kg)</th>
<th>Height (cm)</th>
<th>Blood group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Education

<table>
<thead>
<tr>
<th>Education</th>
<th>Under 4 years</th>
<th>4-10 Years</th>
<th>High school</th>
<th>Undergraduate</th>
<th>Postgraduate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Occupation

| Occupation | |
|------------||
| Father     | |
| Mother     | |
Part 4: Family Medical History

4-1 Did any of the participant’s family members have diabetes mellitus?

☐ Yes.
☐ No.

If yes, please specify how many they are?

☐ 1 person ☐ 2 persons ☐ 3 persons ☐ More than 3 persons

What is the relationship between them and the participant?

☐ Brother. ☐ Sister.
☐ Father. ☐ Mother.
☐ Parental grandmother. ☐ Maternal grandmother.

What type of diabetes did they have?

☐ Type 1 insulin-dependent diabetes mellitus.
☐ Type 2 non-insulin-dependent diabetes mellitus.
☐ Gestational diabetes.
☐ Other, please specify if known--------------------------------------------

4-2 Did the child’s mother have diabetes during pregnancy?

☐ Yes.
☐ No.

Part 5: Participant Medical History

5-1 At what age was the child diagnosed with diabetes?

☐ At birth.
☐ More than 1 week to 1 month.
☐ More than 1 month to 2 months.
☐ More than 2 month to 3 months.
☐ More than 3 months to 6 months.
☐ More than 6 months, please specify ☐ years, and ☐ months.

5-2 In which month was the child diagnosed?

<table>
<thead>
<tr>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
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</thead>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5-3 Did the child show any signs or symptoms of diabetes, such as frequent urination, increased thirst, before being diagnosed with diabetes?
- No, he/she was diagnosed on birth.
- No, he/she was diagnosed on a regular hospital visit.
- No, somebody else brought it to my attention.
- Yes, I recognised some of the signs and symptoms.

5-4 Does the child have any other chronic (lifelong) disease(s)?
- No.
- Yes, please tick one of the following.
  - Rheumatic fever.
  - Rheumatoid arthritis.
  - Multiple sclerosis.
  - Systemic lupus erythematosus.
  - Other, please specify if known----------------------------------------------

5-5 Does the child have any food allergy(s)?
- No.
- Yes, please specify ------------------------------------------------------------------

Does the child have any gastrointestinal disease(s)?
- No.
- Yes, please specify ------------------------------------------------------------------

Part 6: Participant Dietary History

6-1 Was the child breastfed?
- No.
- Yes.

If yes, for how long was the child breastfed?
- Less than 1 week.
- More than 1 to 2 weeks.
- More than 2 weeks to 1 month.
- More than 2 weeks to 3 months.
- More than 3 months, please specify [ ] years, and [ ] months.
6-2 How old was the child when it first received bottle-feeding?
□ Less than 1 week.
□ More than 1 to 2 weeks.
□ More than 2 weeks to 1 month.
□ More than 2 weeks to 3 months.
□ More than 3 months, please specify __________ years, and __________ months.

6-3 What type of milk formula did you first introduced to your child?
□ Cows’ milk-based formula.
□ Soybeans-based formula.
□ Fresh cows’ milk.
□ Nutramigen®.
□ Others, please specify---------------------------------------------------------------

6-4 After the child was diagnosed with diabetes; did you change the type of bottle-feeding formula?
□ No.
□ Yes.
If so, what is the type of the new bottle formula that you have introduced after the diagnosis?
□ Cows’ milk-based formula.
□ Soybeans-based formula.
□ Fresh cows’ milk.
□ Nutramigen®.
□ Others, please specify---------------------------------------------------------------

6-5 How old was the child when it first received an extra food supplement?
□ 3 months
□ 3-6 months
□ 6-9 months
□ More than 9 months, please specify __________ years, and __________ months.

6-6 What is the type of the extra solid food supplement you first introduced to your child?
□ Wheat
□ Rice
□ Soybeans
□ Others, please specify ---------------------------------------------------------------
6-7 What was the procedure you used to prepare your child bottle milk formula?

☐ Boil water then allow cooling and then adding the milk powder
☐ Adding milk powder to cold water and then boil them together
☐ Adding milk powder to cold water and then heating in microwave
☐ Others, please specify ---------------------------------------------

Do you have any extra information you want to add?

--------------------------------------------------------------------------------------------------------
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Thank you for your time
10.2 Appendix B: Information sheet, consent, and survey (Arabic)

10.2.1 Participant information sheet (Arabic)

نموذج (1) بطاقة التعريف بالمرض وطبيعة الدراسة

الأثر المحتمل للبروتينات الغذائية على تطور مرض السكري المعتدل على الأطفال الأردنيين

الباحثون: حيدر الدومي، د. مارك جونز، د. جيم بيرجس. جامعة غرب سدني / أستراليا

ملاحظة

وافقت لجنة أخلاقيات البحوث الإنسانية في جامعة غرب سدني / أستراليا على إجراء هذا البحث. إذا كان لديك أي ملاحظات أو تحفظات حول أخلاقيات البحث العلمي فإنه يمكنك الاتصال بمنسق أعمال اللجنة على تلفون (0061 – 2) 45701136.

الاستفسارات

إذا كان لديك أي استفسارت تتعلق بالدراسة فإنا نرجو أن لا تتردد في الاتصال معنا في أي وقت

Richmond Campus, K-12, Penrith DC, Locked bag 1797, NSW 1797, Australia
الاردن (636), 0796498736, 0616, 96875816, 405543209 (0061–2–1–616)

شكرًا لكم حسن تعاونكم
النمو خلال فترة الرضاعة

يساهم الطعام في تغذية كلاً من عمليتي النمو العضوية والشخصية لدى الرضيع. الأطفال أو المراهقين. إن الأطعمة وعمليات تناولها خلال سنوات الطفولة المبكرة لا يمكن فصلها عن عمليتي النمو والتطور وذلك لأن العملية ككل تشكل الإنسان ككل. إن معظم الأطفال الرضع يتضاعف وزنهم مرتين تقريبا عند بلوغهم السنة الأولى، وثلاث مرات عند بلوغهم العام وذلك يعود إلى تساور نموهم خلال هذه الفترة العمرية الحساسة.

إن الطعام المثالي الأول للطفل الرضيع هو حليب الأم حيث أنه يزوده بالعناصر الغذائية الأساسية والكميات المناسبة لنموه وتطوره بأفضل حال. إن النمو والتطور الإيجابي للأطفال الأصحاء يعتمد على تزويدهم بالغذاء المثالي. ومع ذلك فإن حفاظهم الغذائي تتباين في كل مرحلة من مراحل نموهم. ومع أن حليب الأم هو الغذاء المثالي والمقضي للنوم السريع للأطفال في السنة شهور الأولى فإن إضافة أغذية مساعدة صلبة فيما بعد ذلك يعتبر ضروريًا لنموه وتطوره.

الرضاعة الطبيعية

يمكن للرضاعة الطبيعية أن تتم بنجاح لدى معظم الأمهات اللواتي يحاولن ذلك وخصوصا إذا ما تناولن الأغذية المناسبة لزيادة درار الحليب. وينصح أن تأتي الأم بذكاء بلترتكب أخطار أثناء فترة الحمل، كما وأن حليبها يتطور ليتلامس مع تغيرات حاجات طفلها الغذائية خلال مراحل نموه وتطوره.

إيجابيات الرضاعة الطبيعية

هناك العديد من الفوائد الفسيولوجية والعملية للرضاعة الطبيعية منها: (1) خفض الإصابة بالالتهابات، (2) خفض الإصابة بأمراض الحساسية، (3) سلامة الأطفال، و (4) غذاء مالم وأقتصادي. إذا لم تختار الأم أن ترضع طفلها أو هناك ظروف صحية تحول دون ذلك سواء عند الأم أو عند الطفل فإن حليب البوذرة المصنع من حليب الأبقار هو بديل مقبول.

مرض السكري

يتصف مرض السكري بارتفاع معدلات جلوكوز الدم لدى المصابين. وذلك ينتج عن عدم كفاية أو نقص في إفراز الأنسولين. إن مرض السكري المعتدل على الأنسولين هو أحد أنواع مرض السكري والذي يحتاج فيه المريض إلى المعالجة بحق الأنسولين. ومع أنه عادة ما يظهر في مراحل الطفولة الأخيرة، إلا أنه قد يظهر في أي مرحلة عمرية. كما وأن ظهور هذا المرض في عائلات دون غيرها قد يدل على وجود علاقة وراثية بالمرض.
إن معظم حالات السكري المعتمد على الأنسولين تبدأ باضطرابات في الجهاز المناعي الذاتي لدى الفرد. حيث يشتبه الجهاز المناعي على وجه التحديد في وجود جسم ذاتي يُسمى البروتينات الغذائية، والذي قد يؤدي للإصابة بأمراض مناعية. ويتضمن ذلك (الحمى القرمزية، التهاب المفاصل الروماتيزمي، التصلب العصبي المتعدد والحمى الذئبية).

إن مثل هذا الاضطراب المناعي الذاتي قد يؤدي إلى إنتاج خلايا البكتيريا المسؤولة عن إفراز الأنسولين، ويُشك في ذلك ذلك كلا من البكتيريا ببعض البروتينات أو بعض البروتينات الجاذبة. ويتضح النتائج لذلك أن الفقار ينشأ في إفراز الأنسولين وعندما تبدأ الإصابة السريرية بالمرض، حيث يحتاج المريض إلى العلاج بحق الأنسولين.

وحيث أن حليب الأطفال المصنوع من حليب الفقار يعتبر أول غذاء خارجي يتراوح الطقل الرضيع مع أو بعد فترة الرضاعة الطبيعية، فإن البروتينات حليب الأبقار قد خضعت للبحوث العلمية المكثفة، وتجدر الإشارة إلى أنه لم يتم التوصل حتى الآن إلى أي علاقة سلبية مؤكدة بين تناول تلك البروتينات الغذائية وبين الإصابة بالسكري المعتمد على الأنسولين (المناعي)، كما أنه لم يتم التنبؤ من ذلك علميا.

 مدى انتشار مرض السكري بين الأطفال الأردنيين

بالرغم من النقص في مستويات رعاية وعلاج المصابين بهذا المرض إلا أنه يبقى واحدا من الأسباب الرئيسية لارتفاع كلا من معدلات الكبار والأطفال على المستوى العالمي. وتشير النتائج الإحصائية الحديثة حول مدى انتشار هذا المرض إلى أن هناك أكثر من منة ألف مصاب في منطقة الشرق الأوسط بفترة من الإصابة بالمصاب السكري في الأنسولين، وأن هناك ما يقارب (60000) حالة إصابة تظهر سنويا من بينهم حوالي (3000) طفل تحت سن الخامسة عشرة.


أهداف الدراسة

إن الإفراز السنوي في معدلات الإصابة بهذا المرض بين الأطفال الأردنيين قد دفع الباحث إلى دراسة هذه الظاهرة والتحقيق فيما إذا كان هناك علاقة من عدمها بين تناول البروتينات الغذائية لدى فئة عمرية معينة وبين تطور المرض لديهم.
وعليه فإن الهدف الرئيسي لهذه الدراسة يتلخص في تحديد التغيرات التي قد تؤدي إلى إتمال خلايا البنكرياس المسؤولة عن إفراز الأنسولين وعلاقتها بالإضطرابات المناعية الذاتية لدى أفراد عينة الدراسة، وذلك باختبار أثر الدور المحتمل لبروتينات حليب الأبقار على إحداث مثل تلك الإضطرابات المناعية الذاتية في بنكرياس الأطفال الأردنيين المصابين بهذا المرض، ومقارنة ذلك بغير المصابين بالمرض، وذلك ليس فقط من أجل التقليل من أو تجنب حدوث التلف في خلايا البنكرياس المسؤولة عن إفراز الأنسولين ولكن أيضاً بالنسبة في الوقاية من احتمال الإصابة بهذا المرض.

أغراض الدراسة

إن أغراض الدراسة الرئيسية تتمثل في التالي:

- بناء نمط غذائي عام للأطفال الأردنيين المصابين بالمرض ومقارنتهم بالأطفال الأصحاء، وكذلك
  دراسة التغيرات المحتملة في أساليب تغذيتهم في حالة الإصابة بالمرض.
- دراسة الأثر المحتمل لبروتينات حليب الأبقار وأثره - إذا ما كان هناك - على تطور المرض.
  وذلك من خلال دراسة مستويات الأجسام المضادة في اللعب و مصل الدم لدى الأطفال المصابين بالمرض ومقارنتهم بأفراد غير المصابين.

أهمية الدراسة

إن أهمية هذه الدراسة تكمن في تحسين فهمنا للمرض، وعلى فعليه فإن مشاركتك في هذه الدراسة تسهم مساهمة فعالة في تمكن الباحثين في هذه الأسباب الكامنة وراء الإزدياد في معدلات الإصابة بالمرض على المستوى العالمي والمحللي. تلك إضافة إلى أن فهمنا لهذا الظاهرة سيساعد في إيجاد وسائل الوقاية المناسبة ليس فقط للتفتيت من الإصابة بالمرض بل أيضاً للحد منها.

دورك في الدراسة

إن موافقتك على أن يكون إنك / إنكما من ضمن أفراد عينة الدراسة يستدعي منك التوقيع على نموذج الموافقة على المشاركة بالدراسة (نموذج 3).

أن تجرب على أسئلة الاستبيانة (نموذج 2)، والذي سيساهم في بناء نمط غذائي عام للطفل الأطفال، وكذلك دراسة التغيرات المحتملة في أساليب تغذيتهم إذا ما أصابوا بالمرض، ومقارنة ذلك بالأطفال غير المصابين بالمرض.

السماح بجمع عينة من عينة الطفل، والذي سيكون الباحث من تحديد الأجسام المضادة التي قد تنشأ ضد بروتينات حليب الأبقار، يرجى مراقبة أن لا يتناول الطفل أي طعام قبل ثلاثين دقيقة من موعد أخذ العينة وكذلك مضيفة فم الطفل بالماء.
لمقارنة الأجسام المضادة التي قد تظهر في عينات الطفل مع تلك التي قد تكون في الدم. إنه وبعد موافقتهم
سيقوم شخص مؤهل بسحب عينة دم من الطفل / الطفلة.

بشكل أساسي فإنه سيتم جمع عينة واحدة فقط من الطلاب و/ أو عينة واحدة فقط من الدم.

بعد موافقتهم. فإنه و دراسة التغييرات المحتملة لمعدلات الأجسام المضادة لدى الأطفال المشحونين حديثا
بالمرض أو يظاهرون أن لهم خلال فترة إجراء الدراسة، وكذلك دراسة التغييرات المحتملة في أنماط تغذيتهم
في أي فترة عمرية خلال الدراسة فإنه سيتم جمع عينة إضافية من الطلاب و/ أو الدمناء.

بعض الآثار السلبية

لتلاقى حدوث أي آثار سلبية أثناء عملية سحب عينة الدم فإن فني مختص سيقوم بجمع تلك العينة، ومع ذلك فإن
منطقة إجبار خفيفة قد تظهر مكان وخد الإبرة. هذا مع العلم أن عملية سحب عينة الدم ستجري في غرفة
مجهر لذا الغرض، وستستخدم أكبة سحب ذات الاستخدام لمرة واحدة. وكذلك سيتم تطهير مكان الوخز بالإبرة
بشكل علمي.

- إذا ما فشل فني سحب عينة الدم في سحب العينة في أول مرة فإنه لن يتم السحب مرة ثانية إلا بعد
موافقة والتوقيع مرة أخرى على نموذج الموافقة على المشاركة بالدراسة (نموذج 3).

- قد يشعر الطفل أثناء سحب عينة الدم بعدم الراحة و/ أو الخوف، وعليه فإنه سيطلب من الوالدين
المساهمة في تهدئة وطمأنة الطفل.

- سيستلم فني سحب العينات كائن كل جهد فتى ممكن لسحب العينة بهدوء وسلاسة وستكون عرفة السحب
مريحة وذات جاهزة عالية.

- أما فيما بين سحب عينة اللعب فإنه ليس من المتوقع أن يؤدي ذلك إلى أي توتر أو آثار سلبية، إلا أن
الطفل قد يشعر بشيء من عدم الراحة. وعليه فإنه سنعمل جاهدين ومساعدات الوالدين لأن يكون المشارك
مسترخياً وهداناً.

الخصوصية والسرية

ستحافظ الباحث على أقصى درجات السرية والخصوصية المتعلقة بالمشارك والمشارك وكذلك البيانات المتعلقة به
لن يتم التعرف بأي شخص مشارك خلال أو بعد إجراء الدراسة أو الإشارة إليه عند نشر نتائج الدراسة.
إن جميع الوثائق المتعلقة بالشخص المشارك بما فيها نموذج الموافقة على المشاركة بالدراسة (نموذج 3) سيتم حفظها خلال فترة الحفظ الإلزامية في مكان آمن ومنفصل عن البيانات المتعلقة بالمشارك وذلك وفقا لمعايير جامعة الباحث حيث سيتم إبلاغها رسميا بعد ذلك. يذكر أنك لك مطلق الحرية للإمتناع عن المشاركة أو الانسحاب من هذه الدراسة في أي وقت تشاء. هذا مع العلم بأنه لن يكون هناك أي مخاطر أو تبعات جزائية أو غير جزائية أو أي نتائج عكسية تترتب على إمتناعك أو الانسحاب من المشاركة بالدراسة.

نتائج الدراسة

بناءً على رغبتك فإنه سيتم تزويدك بنتائج البحث حال ظهورها مع العلم بأن نتائج هذا البحث سيتم نشرها في المجلات العلمية المحلية والعالمية.

يمكنك الاحتفاظ بهذا الملخص إضافة إلى صورة عن نموذج الموافقة على المشاركة بالبحث.

شكرًا لكم حسن تعاونكم
نموذج (2) الموافقة على المشاركة بالدراسة

عنوان الدراسة: الأثر المحتمل للبروتينات الغذائية على تطور مرض السكري المعتد على الأنسولين بين الأطفال الأردنيين.

باحث: حيدر عبد الله الدومي

أعلم أن السيد حيدر الدومي هو طالب دكتوراه. يدرس: (الثر المحتمل للبروتينات الغذائية على تطور مرض السكري المعتد على الأنسولين بين الأطفال الأردنيين). حيث أن الباحث سيجري هذه الدراسة وفقاً لمعايير أخلاقية ومبادئ البحوث العلمية المحددة من قبل لجنة أخلاق البحوث الإنسانية في جامعة غرب أستراليا.

أقر بأنني قد قرأت أو قرأ لي بطاقة التعريف بالمرض وطبيعة الدراسة (نموذج 1) ويني قد قررتها.

وتعتبر كذلك على أذى الإحصاء للدراسة العامة، أساليبها، متطلباتها، والأثر السلبي المحتملة والتي قد تظهر أثناء إجراء الدراسة، وأنه قد تجربا شرحنا لي من قبل: ................................................................. (الباحث).

وحيث أن ابني/ ابنتي تحت سن الرابعة عشرة فإني أقر بأنه وكوني ولي أمره/ أرها فإنه يتوجب

على التوقيع بالموافقة على المشاركة بالدراسة (نموذج 3).

أقر بأنني قد منحت الوقف الكافي لدراسة المعلومات المتوفرة، وكذلك لطلب التصيحة اللازمة إذا ما

رغبتك بذلك.

أدرك بأن هذه الدراسة ستستغرق ما يقارب العشرون (20) دقيقة من وقتك ومن ضمنها الوقف اللازم لتعين الاستبانة (نموذج 2).

أقر بأنني على دراية بأذى الإحصاء للدراسة العامة، أساليبها، متطلباتها، والأثر السلبي المحتملة، والتي قد تظهر خلال الدراسة.

أقر بأنني أعرف بأن المشاركة بهذه الدراسة قد تسبب بعض الآثار الجسدية أو/و النفسية السلبية لطفي أو/و لطفي.

أقر بأن مشاركتي في هذه الدراسة هي طوعية بحثيا وأنه يحق لي الامتناع أو الانسحاب من الدراسة في أي وقت.

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أعرف بأنه يحق لي الانسحاب من الدراسة في أي وقت أثناء وأن ذلك الانسحاب لن يؤثر على مستوى الرعاية الصحية / الطبية المقدمة لي أو لأي من أفراد عائلتي، وأنه بذلك لن يؤثر على طبيعة علاقتي بالفريق الطبي المعالج.

أعلم بأن هذا البحث قد أقر من قبل لجنة أخلاقيات البحوث الإنسانية في جامعة غرب سدني/ أستراليا.

أقر بأنني قد استلمت نسخة من هذا النموذج إضافة إلى طبيعة الدراسة (نموذج 2).

(نموذج 2)

إذا ما كان لى أي تساؤلات حول الدراسة أو المشاركة فيها فإننى أعلم بأنه يمكنني الاتصال بالباحث (حذير الدموي) على تلفون (0796498736 / الأردن) (04054543209 / أستراليا).

وافق على المشاركة بهذه الدراسة وقد تأكدت بأنه سيتم اتخاذ أقصى درجات السرية الشخصية، وخصوصية البيانات. وأنه لن يتم الإفصاح نهائياً عن هويتي أو هوية المشاركات / المشاركة سواء أثناء الدراسة أو بعد نشر نتائجها.

لقد تم إخطارى بوضوح بأن جميع الوثائق المتعلقة بالإستبانة (نموذج 2) ونموذج الموافقة على (3) المشاركة بالدراسة (نموذج 3) والموقع على أنه سيتم محتضها في مكان آمن وسريع منفصلا عن بقية البيانات المتعلقة بالدراسة للدولة الإرامية ومدتها خمس سنوات والمصول بها في جامعة غرب سدني / أستراليا. وبعدها سيتم إتلافها وفقا ل نظام الجامعة.

المشارك بالمشاركة

الاسم / الشهادة / من:.............................................

العنوان / الملاحظة:.............................................

تاريخ الولادة:.............................................

توقيع المشاركة / المشاركة (إذا كان ذلك ممكنًا):.............................................

تاريخ التوقيع:.............................................

اسم والأدانة الطالب / الطالبة المشاركة:.............................................

توقيع والآدار / الدائرة المشاركة:.............................................

تاريخ:.............................................

توضيح الشهادة:.............................................

التاريخ:.............................................
نموذج 3: الاستبانة

تحية طيبة وبعد: -

استكمالاً لتطابقات درجة الدكتوراه في مجال سكري الأطفال سيقوم الباحث بدراسة المناعي تحت عنوان "الأثر المحتمل للبر وتينات الغذائية على تطور مرض السكري المعتمد على الأنسولين لدى الأطفال الأردنيين". هذا مع العلم أن لجنة أخلاقيات البحوث الإنسانية في جامعة غرب سدني (UWS-HERC/HEC 02/162) قد وافقت على إجراء هذه الدراسة.

أخي الكريم / أختي الكريم: -

قبل البدء بتعيين الاستبانة بين يديك يرجى مراعاة التالي:
- قراءة بطاقة التعريف بالمرض وطبيعة الدراسة (نموذج 1) والاحتفاظ بنسخة إضافية لنفسك.
- التوقع على نموذج الموافقة على المشاركة بالدراسة (نموذج 2) والاحتفاظ بنسخة إضافية لنفسك.
- وارسال الأخرى للباحث.
- تعبئة الاستبانة (نموذج 2) والمكونة من ستة أجزاء وإعادتها للباحث.
- يمكنك وضع إشارة X في المربعات أمام الفقرة المناسبة أو الكتابة حسبما تراه مناسباً.

إذا كان لديك أي أسئلة أو استفسارات أو كان لديك الرغبة في معرفة المزيد عن هذه الدراسة ونتائجها فإنه يمكنك الاتصال بالباحث على العنوان أدناه.

شكرًا لكم حسن تعاونكم ومؤكداً أن هذا البحث ما هو إلا مساهمة متواضعة في مجال الكشف عن أسباب هذا المرض والتحديث منها.

وتقبلوا الاحترام

الباحث
جدير عبد الله الحموي (الأردن/موبايل) 0796498736 (سدني/موبايل) 61-2-96875816
البريد الإلكتروني h.aldomi@uws.edu.au / haldomi@yahoo.com
العنوان البريدي Richmond Campus, K-12, Locked Bag 1797 DC, NSW 1797 Australia
الجزء 1: معلومات عامة

اسم الطفل/ الطفلة (اختياري).................................................................

اسم الأم (اختياري).................................................................

العنوان (اختياري).................................................................

الآباء (اختياري).................................................................

أرجو وضع إشارة عند الجواب المناسب أو الكتابة حسبما تراه مناسبًا.

من هو الشخص الذي سيقوم بتعينه هذه الاستبانة؟
- أم المؤمنين
- بأب المؤمنين

هل لديك طفل/ طفلة مصابة بالسكري؟
- نعم
- لا

إذا أجبت (لا) اذهب إلى الفقرة 2.

إذا أجبت (نعم) على السؤال أعلاه، فهل توافق على:
- تعيني Weather
- السماح بأخذ عينة لصاب
- السماح بسحب عينة دم

هل ترغب أن يكون ابنك/ ابنتك من ضمن أفراد عينة الدراسة من غير المصابين بالسكري؟
- نعم
- لا

إذا أجبت (نعم) على السؤال 2، فهل توافق على:
- استبانة تعين
- السماح بجمع عينة لصاب
- السماح بسحب عينة دم

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المشاركة/الجزء 2: بيانات عامة عن المشارك

<table>
<thead>
<tr>
<th>الترتيب</th>
<th>الوزن عند الولادة (كغم)</th>
<th>الطول (سم)</th>
<th>العمر (شهر/سنة)</th>
<th>الجنس</th>
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فضيلة دم المشارك/ المشاركة

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<td>O-</td>
<td>O+</td>
<td>B+</td>
<td>B-</td>
<td>AB+</td>
</tr>
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<td>AB-</td>
<td>A-</td>
<td>A+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

الجزء 3: بيانات عامة عن الآباء/الأمهات

<table>
<thead>
<tr>
<th>فضيلة الدم</th>
<th>العمر (شهر/سنة)</th>
<th>الوزن (كم)</th>
<th>الطول (سم)</th>
<th>الأم</th>
<th>الأب</th>
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التعليم

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المهنة

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</tbody>
</table>
جزء 4: تاريخ العائلة الطبي

1- هل هناك أحد في أفراد عائلة المشارك مصاب بالسكري؟

نعم لا

إذا كان الجواب نعم، أرجو تحديد العدد. شخص واحد أشخاص أكثر من 3 أشخاص

ما صلة قراءة أفراد العائلة المصابين بالمشارك؟

أب أم أخ جد من طرف الأم جد من طرف الأب

ما نوع السكري لدى أفراد العائلة المصابين؟

سكري معتدل على الأنسولين
سكري غير معتدل على الأنسولين
سكري حمل نوع آخر، أرجو التحديد:

2- هل أصيبت والدة المشارك بالسكري أثناء الحمل؟

نعم لا

الجزء 5: التاريخ الطبي للمشارك/المشاركة

1-5 إذا كان الطفل/ الطفلة مصابا بالسكري ففي أي سن تم التشخيص؟

١-٥ سنوات عدم الولادة
من أسبوع إلى شهر واحد
من شهر إلى شهرين
من شهرين إلى 3 شهور
من 3 شهور إلى 6 شهور
أكثر من 6 شهور، أرجو تحديد السنة والشهر
5-2 إذا كان الطفل / الطفلة مصابا بالسكري. في أي شهر تم التشخيص؟

<table>
<thead>
<tr>
<th>كانون أول</th>
<th>كانون ثاني</th>
<th>كانون أول</th>
<th>تشرين ثاني</th>
<th>تشرين أول</th>
<th>أيلول</th>
<th>تموز</th>
<th>آب</th>
<th>حزيران</th>
<th>يونين</th>
<th>أيار</th>
<th>نيسان</th>
</tr>
</thead>
</table>

5-3 إذا كان الطفل / الطفلة مصابا بالسكري، هل أظهر الطفل قبل تشخيص المرض أي أعراض للإصابة بالسكري (مثل تكرار البول، زيادة العطش)؟

- لا، تم فحصه عند الولادة
- لا، تم تشخيصه خلال زيارة طبية منتظمة للمستشفى / المركز الصحي
- لم تلاحظ شخص آخر لنع، فقد لاحظت بعض الأعراض على الطفلة / الطفل

5-4 هل يعاني الطفل / الطفلة من أي أمراض مزمنة أخرى؟

- لا
- نعم. أرجو وضع إشارة عند المناسب

- الحمى القرمزية
- التهاب المفاصل الروماتيزمي
- التصلب العصبي المتعدد
- الحمى الذوبية
- أي أمراض أخرى. أرجو التحديد: ...

5-5 هل للطفل / للطفلة أي حساسية ضد طعام معين؟

- لا
- نعم. أرجو التحديد: ...

5-6 هل يعاني الطفل / الطفلة من أي أمراض متعلقة بالجهاز الهضمي؟

- لا
- نعم
المشاركة/جزء 6: التاريخ الغذائي للمشارك

1- هل رضعت الطفل/الطفلة رضاعة طبيعية؟
   - لا
   - نعم

إذا كانت الإجابة (نعم) فما هي المدة التي رضعها / رضعتها رضاعة طبيعية؟
   - أقل من أسبوع
   - أكثر من أسبوع إلى أسبوعين
   - أكثر من أسبوعين إلى شهر
   - أكثر من أسبوعين إلى 3 شهور
   - أكثر من 3 شهور

2- كم كان عمر الطفل/الطفلة عند أول استخدام للفولتية (الرضاعة)؟
   - أقل من أسبوع واحد
   - من أسبوع إلى أسبوعين
   - أكثر من أسبوعين إلى شهر واحد
   - أكثر من 3 شهور. أرجو تحديد السنة والشهر

3- ما نوع الحليب الذي فتم للطفل/الطفلة كدبيل عن حليب الأم؟
   - حليب بودرة مصنعة من حليب البقر
   - حليب بودرة مصنعة من فول الصويا
   - حليب بقر طازج
   - حليب بودرة للوقاية من أثار الحساسية

   أرجو تحديد النوع الآخر:

4- بعد اكتشاف الإصابة بالسكري (إذا كان الطفل / الطفلة مصابا بالسكري)، هل غيرت نوع حليب البودرة؟
   - نعم
   - لا

إذا كنت قد غيرت نوع الحليب، ما هو نوع الحليب الجديد الذي قدمته؟
   - حليب بودرة مصنعة من حليب البقر
   - حليب بودرة مصنعة من فول الصويا
   - حليب بقر طازج
   - حليب بودرة للوقاية من أثار الحساسية

   أرجو التحديد:
5- كم كان عمر الطفل/الطفلة عندما بدأ يتناول أطعمة إضافية صلبة بجانب الحليب؟

- ثلاثة أشهر
- من ثلاث إلى ستة أشهر
- من ستة أشهر إلى تسعة أشهر
- أكثر من تسعة أشهر. أرجو تحديد السنة والشهر

6- ما نوع أول طعام إضافي صلب قدمته لطفلك؟

- قمح
- أرز
- فول الصويا
- أنواع أخرى. أرجو التحديد:

7- ما الطريقة التي استعملت في تحضير زجاجة الحليب؟

- على الماء ثم الانتظار لحين بروزه ثم إضافة الحليب إليه
- إضافة حليب البوذرة للماء البارد ثم غليهما معا
- إضافة حليب البوذرة للماء البارد ثم تخزينهما بالميكرويف
- طرق أخرى. أرجو التحديد:

هل هناك أي معلومات إضافية ترغب بذكرها؟

شكرا لتعاونكم