Aus der Poliklinik für Zahnerhaltung, Parodontologie und Endodontologie
Westdeutsche Kieferklinik
Heinrich-Heine-Universität Düsseldorf
Stellv. Direktor: Univ.-Prof. Dr. med Dr. med.dent Thomas Beikler

Investigation of the oral status and microorganisms in children with phenylketonuria and type 1 diabetes

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Preeti Singh-Hüsgen

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Referent: Univ.-Prof. Dr. W. H.-M Raab

Korreferent: Priv.-Doz. Dr. Thomas Meißner

This thesis is dedicated to my children,

Arjun and Agastya

--Unknowingly supportive in their love, daring to dream!

Abstract

The aim of this study was to investigate the oral parameters that influence the caries risk and risk of developing periodontal disease in children with phenylketonuria (PKU) and type 1 diabetes. The parameters to be assessed were the dental and oral hygiene status, gingival health and oral microorganisms in children with diabetes, PKU and healthy children.

Material and methods: Children between the ages of 3 and 18 years were recruited for each group in the study. The total sample consisted of 238 children. The PKU group had 38 children and both the diabetic and healthy control group comprised of 100 children each. Demographic data was collected with the help of a standard questionnaire. The dental status was assessed by the dmfs / DMFS Index and the gingival health and oral hygiene was evaluated using the Papillary Bleeding Index (PBI) and the Silness & Löe Index. For the microbiological examination of the bacteria, a sample was collected on a sterile paper point from the cervical region and gingival sulcus of the first deciduous molar or from the first permanent molar in the lower right quadrant. Quantitative real-time polymerase chain reaction was used for the detection of Streptococcus mutans (Sm), Lactobacillus casei (Lca), Lactobacillus species (Lac), Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg), and Tannerella forsythensis (Tf). The level of metabolic control of diabetes mellitus for the children with diabetes was determined by the glycosylated haemoglobin value.

Results: A statistically significant difference in the dmfs Index value was found between the three groups. The mean dmfs index value for the PKU children (4.18) was found to be relatively high as compared to the other two groups. No statistically significant difference was found in the mean DMFS values between the children and adolescents in the three groups. The diabetics showed statistically significant higher values for the Silness and Löe Index than the other two groups. The diabetic group also revealed a small statistically significant difference in the PBI score.

A comparison of all the bacteria between the three groups revealed a statistically significant difference between Lac, Lca and Pg. Counts of Lac were found to be the lowest in the PKU children. The diabetics showed the highest counts of Lca but lowest for Pg. The healthy children showed the lowest counts for Lca but highest counts for Lac and Pg.

Conclusions: Children with PKU possess a higher caries rate in their primary dentition. While diabetic children have a lower caries rate in their primary dentition, they show a higher risk of developing periodontal disease. It is, therefore, proposed that both groups of child patients, i.e. PKU and type 1 diabetics be encouraged to seek early dental advice and be incorporated in a meticulous prevention programme.

Abbreviations

µg	Microgram
µl	Microlitre
µmol	Micromole
Aa	Agregatibacter actinomycetemcomitans
ATCC	American Type Culture Collection
BH4	Tetrahydrobiopterin
C	Celsius
C_t	Cycle threshold
DMFS	Decayed, missing, filled surfaces for the dentition
dmfs	Decayed, missing, filled surfaces for the primary dentition
DNA	Desoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HbA1c	Glycated hemoglobin
HLA	Human leukocyte antigen
IDF	International Diabetes Federation
IgA	Immunoglobulin A
IL-1b	Interleukin 1 beta
ISPAD	International Society for Pediatric and Adolescent Diabetes
kb	Kilobase
L	Litre
Lac	Lactobacillus species
LB-Amp	Lysogeny Broth (LB) containing ampicillin
Lca	Lactobacillus casei
min	Minute
ml	Millilitre
mM	Millimol
mM	Millimolar

mmol	millimole
MMP-8	Matrix metalloproteinase-8
ng	Nanogramm
nm	Nanometre
nt	Nucleotides
OD	Optical Density
OPA	One Phor All
PAH	Phenylalanine hydroxylase
PBI	Papillary Bleeding Index
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pg	Prophyromonas gingivalis
Phe	Phenylalanine
PKU	Phenylketonuria
PMN	Polymorphonuclear
RNA	Ribonucleic acid
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
secs	Seconds
Sm	Streptococcus mutans
TBE	Tris-Borate-EDTA
Tf	Tannerella forsythensis
Tris	Tris(hydroxymethyl)aminomethane
Tyr	Tyrosine
WHO	World Health Organisation

Index

1 Introduction

It is often essential for children suffering from metabolic diseases to follow a strict diet to keep the disorder under check and to be able to develop and function normally. Phenylketonuria (PKU) is a metabolic disorder in which the patients present with an absence or deficiency of the enzyme phenylalanine hydroxylase which is essential to metabolise the amino acid phenylalanine into the amino acid tyrosine. Uncontrolled, the disease can lead to the accumulation of phenylalanine in the blood and brain causing disabilities. In order to keep the ingestion of phenylalanine to a minimum, children with PKU follow a special low protein diet. At the same time, their diet is rich in carbohydrates and the phenylalanine -free formula drinks have a high pH (Kilpatrick et al., 1999). The frequency of ingestion of these carbohydrates is high and therefore, the risk for the development of caries in children suffering from PKU is considered to be high.

Diabetes is another metabolic disorder, caused due to defective insulin secretion and action resulting in abnormally high blood glucose levels. The disease is principally classified into type 1 and type 2 diabetes. Traditionally, children suffering from type 1 diabetes had to follow a diet restricted in carbohydrates (Goteiner et al., 1986) in order to maintain normal blood sugar levels. The intake of carbohydrates was aligned with the insulin regime. Advancements in insulin therapy regimes have led to the relaxation of dietary restrictions and type 1 diabetics are now able to follow a diet quite similar to normal healthy individuals (Ciglar et al., 2002). Furthermore, due to alterations in the polymorphonuclear (PMN) leucocyte function, collagen metabolism and a possible alteration in the microbial flora, it has been found that type 1 diabetics are more prone to infections than healthy individuals. Earlier studies have shown that although children with type 1 diabetes possess a lower caries risk, they have an increased risk of developing periodontal disease (Goteiner et al., 1986; Firatli, 1997). Several studies have made diabetes and oral health their

subject to elucidate the impact of both on each other. A large portion of these studies have, however been conducted on type 2 diabetic patients. Studies on children have documented inconsistent results which may be due to the varying susceptibility of the patients and the multifactorial nature of both diseases (Karjalainen, 2000).

The present study aims to provide an insight into the oral status of children suffering from two different metabolic diseases. At the same time it seeks to answer questions on whether the dietary regulations of the children have an effect on their dental health and whether there is an alteration in their oral microflora, putting the children at a higher or lower risk for developing dental caries and periodontal disease.

2 Literature Review

2.1 Diabetes

The ISPAD (International Society for Pediatric and Adolescent Diabetes) has defined Diabetes as follows:

“Diabetes is a group of metabolic diseases characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The abnormalities in carbohydrate, fat, and protein metabolism that are found in diabetes are due to deficient action of insulin on target tissues. If ketones are present in blood or urine, treatment is urgent, because ketoacidosis can develop rapidly.” (Craig et al., 2009)

Patients with hyperglycemia present with the symptoms of polyuria, polydipsia, blurred vision and weight loss associated with polyphagia. Chronic hyperglycemia may be accompanied by impairment of growth and susceptibility to certain infections. Inadequacy due to impairment of insulin secretion and defects in insulin action may exist in the same patient, making it difficult to define which of the above is truly responsible for the hyperglycemia.

Diabetes can be mainly classified in type 1 and type 2 diabetes. Further classification of diabetes includes cases of hyperglycemia due to genetic defects of the β-cells or in insulin action, endocrinopathies, infections, induced by drugs or chemicals, pancreatic diseases, associated with syndromes and gestational diabetes.

Patients with type 1 diabetes (insulin dependent diabetes or juvenile-onset diabetes) have an absolute deficiency of insulin secretion. This deficiency is mainly due to T-cell mediated pancreatic islet β-cell destruction. It is believed that environmental factors can also trigger pancreatic cell destruction. However, these triggers, chemical or viral, are still unknown. The disease becomes clinically evident after approximately 90% of the pancreatic β-cells have been destroyed. Further, it has been found that a susceptibility to autoimmune type 1 diabetes can be transmitted by multiple genes, the chief gene being the Human

leukocyte antigen (HLA) gene. Individuals at risk of developing the disease can be identified by an intravenous glucose tolerance test and measurement of autoantibodies and genetic markers associated with type 1 diabetes.

Patients with type 2 diabetes (non–insulin dependent diabetes or adult-onset diabetes) show a combination of resistance to insulin action, usually coupled with a relative insulin deficiency. The specific etiology of the disease is unknown but patients do not show an autoimmune destruction of β-cells or any other cause of diabetes. These patients are usually obese and obesity itself leads to a certain degree of insulin resistance. Others who are not obese usually exhibit an increased percentage of body fat distributed primarily in the abdominal region. Hyperglycemia develops gradually and remains undetected for a long time as it is not severe enough to present the classic symptoms of diabetes. Although undetected, this hyperglycemia is sufficient to cause pathologic and functional changes in various target tissues (American Diabetes Association, 2010).

2.2 Diagnosis of diabetes

Diagnosis of diabetes is based on blood glucose measurements and the presence or absence of symptoms and can be established by the following criteria:

1. Symptoms of diabetes plus casual glucose concentration ≥ 11.1 mmol/L (200 mg/ dl)
2. Fasting plasma glucose ≥ 7.0 mmol/L (≥ 126 mg/dl)
3. 2-hour post-load glucose ≥ 11.1 mmol/L (200 mg/ dl) during an oral glucose tolerance test
4. HbA1C (glycated hemoglobin) ≥ 6.5

(Global IDF/ISPAD Guideline for Diabetes in Childhood and Adolescence, 2011)

2.2.1 Epidemiology of diabetes

Over 90% of child and adolescent diabetics, in most western countries, suffer from type 1 diabetes. Less than half of these patients are diagnosed before the age of 15 years. The incidence of type I diabetes varies considerably between and even within countries and between different ethnic populations. The highest incidence is found in Finland (64 per 100,000/ year) (Harjutsalo et al., 2008) and the lowest in China (0.1 per 100,000/year) and Venezuela (The DIAMOND Project Group, 2006). Environmental causes are believed to be the reason for this great disparity. A rise of 2 to 5 percent per year has been noted in several parts of the world. This amounts to an astonishing rise of 50% in 10 years (British Medical Association, 2004). This rise shows a disproportionately greater increase in diabetics under the age of 5 years. In Germany, the prevalence of type I diabetes for children under 14 years is 10,000-15,000 children (Ehehalt et al., 2008; Rosenbauer et al., 2002) and 21,000-24,000 (Rosenbauer et al., 2002) for children under 19 years of age.

Gender differences in incidence have also been found in some populations. A seasonal variation in the presentation of new cases is seen, with the peak being observed in winter months.

Type 2 diabetes accounts for approximately 90% of all cases. Social and behavioral changes are considered as the main factors responsible globally for the sudden increase in type 2 diabetes. Type 2 diabetes is being more frequently diagnosed in adolescents in recent years, mostly in the peri-pubertal period (Global IDF/ISPAD Guideline for Diabetes in Childhood and Adolescence, 2011).

In general, population growth, aging, urbanization, and an increasing prevalence of obesity and physical inactivity are leading to an increase in diabetes. An estimated 171 million people were suffering from diabetes in 2000 and this number is anticipated to rise to 366 million people in North America and Europe by 2030 (Wild et al., 2004).

2.2.2 Complications of diabetes

Complications of diabetes can be classified as macrovascular and microvascular. Macrovascular complications encompass atherosclerotic cardiovascular diseases such as coronary heart disease, stroke and peripheral vascular disease. Hyperglycaemia is the chief etiological factor of both these complications. Chronic elevation of blood glucose level leads to the damage of blood vessels due to the increased deposition of surface glycoproteins, which then results in weakness of the basement membrane. The endothelium of the retina, kidney and peripheral nervous system allow entry of glucose even in the absence of insulin, subsequently initiating the microvascular complications known as retinopathy, nephropathy and neuropathy.

One long term study, where patients were followed up for an average period of 6.5 years revealed that intensive therapy, keeping the blood glucose concentrations close to the normal range, effectively delayed the onset, decreased the frequency and severity, and at the same time slowed the progression of diabetic retinopathy, nephropathy, and neuropathy in patients with insulin dependent diabetes mellitus (The Diabetes Control and Complications Trial Research Group., 1993). Some researchers, however, refute the fact that hyperglycemia is the main cause of the diabetic complications, supporting their hypothesis with examples of well controlled diabetics also developing the complications in the course of time (Rich, 2006).

2.3 Type 1 diabetes and oral health

A number of studies have been dedicated to the investigation of oral health in diabetics. The majority of these studies have been focused on the relationship between periodontal disease and diabetes and fewer on dental caries and diabetes. Although the risk of developing periodontal disease in diabetics is well established, the association of dental caries and diabetes is still debated. It is, however, difficult to interpret the significance of the results in relation to children

as a large amount of the studies regarding oral health in diabetics have been carried out with type 2 diabetic adult patients.

2.4 Type 1 diabetes and dental caries

As mentioned above, the association between dental caries and diabetes type 1 has not been clearly established. Earlier studies have categorically shown that children with diabetes had lesser dental caries as compared to healthy children, the reason for this being stated as the restricted carbohydrate (sugar) intake of diabetics. However, more recent studies have reported no difference in the caries incidence of children with type 1 diabetes and their healthy counterparts (Edblad et al. 2001; Moore et al., 2001b; Lalla et al., 2006a). It is believed that the newer treatment regimens have led to a better control of the disease, thus allowing the diabetic children to have a diet quite similar to that of normal healthy children (Tonella et al., 2010). Therefore, recent studies investigating the relationship between diabetes type 1 and dental caries are focusing on the metabolic control of the disease. Longitudinal studies have been able to show a positive correlation between the degree of metabolic control and the development of dental caries (Twetman et al., 1992; Karjalainen et al., 1997; Twetman et al., 2002). Interestingly, a more recent study has been able to illustrate a positive relationship between caries risk and metabolic control, with a sevenfold increased risk of impaired metabolic control after 3 years in those assessed with high caries risk at onset (Twetman et al., 2005).

The multifactorial nature of dental caries makes it difficult to identify an exact factor that could be responsible for an association between dental caries and type 1 diabetes. In order to have a better understanding of this association a brief account of the biological basis of the factors involved is necessary.

It has been postulated that impaired salivary secretion causes slow oral clearance of sugar resulting in changes in the pH of dental plaque and thereby, increasing the risk of caries development (Hase & Birkhed, 1988). Additionally, type 1 diabetics show an alteration in the salivary glands (Reznick et al., 2006)

which causes a change in the composition of saliva and in the salivary flow rate (Ben-Aryeh et al., 1988; Moore et al., 2001a; López et al., 2003; Mata et al., 2004, Busato 2012), and this in turn has an effect on the oral microflora. These changes have been found to remain over a period of 2 years (Siudikiene et al., 2008). Another cross-sectional study confirmed that decreased unstimulated salivary flow rates were associated with high caries levels in diabetic children (Siudikiene et al., 2006).

a. Salivary composition

Glucose

Several studies have shown that type 1 diabetics with poor metabolic control show an increase in the salivary and sulcular glucose levels due to the hyperglycemia (Reuterving et al., 1987), reduced salivary glucose clearance and a disruption in the neuroregulatory mechanism of the salivary glands (Sampaio et al., 2011). Increase in the salivary glucose promotes bacterial growth, decrease in the wound healing capacity and leads to an increase in lactic acid production. This results in a decrease in the oral pH, thereby simultaneously reducing the buffering capacity of saliva and altering the bacterial colonization of the biofilm, in favour of Streptococcus mutans and lactobacillus (Sampaio et al., 2011).

Proteins

Due to its known antimicrobial properties the role of IgA in the development of dental caries has been investigated. However, its influence on caries development has not as yet been substantiated. Siudikiene et al. (2008) showed that children with diabetes had a significantly higher total immunoglobulin A (IgA) and protein concentration over a study period of 2 years. López et al. (2003) found that the saliva of type 1 diabetics showed a more acidic pH, reduced salivary flow rate, an increased level of total sugars, glucose, urea and total proteins, whereas the calcium level was found to be decreased. Other studies have shown no differences in the total concentration of salivary proteins in type 1 diabetics and healthy controls (Edblad et al., 2001).

Isola et al. (2011) demonstrated that Statherin, a salivary protein involved in regulation of calcium homeostasis and the formation of enamel pellicle was found to be in a lower concentration in diabetics. The authors concluded that this might account for the higher incidence of caries and infections in diabetics. On the other hand, Engström and Kirstilä showed that patients with a total IgA deficiency did not have a higher caries incidence than their control subjects (Engström et al., 1992; Kirstilä et al., 1994).

Amylase

Long-chain carbohydrates are broken down by salivary amylase. The alpha-amylases constitute a complex group of salivary digestive enzymes which provide receptors on the tooth surface for the adherence of certain species of Streptococcus thus promoting the formation of plaque and later dental caries. It is, therefore, postulated that patients with uncontrolled type 1 diabetes and elevated alpha-amylase levels, due to a carbohydrate-rich diet, may be more prone to the development of dental caries (Sampaio et al., 2011).

Calcium

The concentration of calcium in saliva has also been investigated as a possible cause in the development of caries. The process of remineralisation of carious lesions can be promoted by increasing the concentration of calcium in saliva. Some researchers have been able to demonstrate a higher concentration of salivary calcium in diabetic individuals (Mata et al., 2004; Moreira et al., 2009), whereas others have reported lower concentrations of salivary calcium in diabetic patients (López et al., 2003). Other authors have found no difference between normal and diabetic children in the concentrations of calcium in saliva (Edblad et al., 2001).

b. Alterations in salivary microbiota

Streptococcus mutans and Lactobacillus are the two main microorganisms involved in the development of dental caries. Streptococcus mutans play an important role in the initiation of the carious lesion and Lactobacillus is responsible for the progression of caries once the lesion has been formed or

demineralisation has taken place. As with healthy subjects, these two microorganisms have been studied in type 1 diabetics. In 1989, Twetman investigated the saliva of 94 children with type 1 diabetes and found that there was no difference in the distribution or number of Streptococcus mutans in healthy and type1 diabetes children, but the diabetic children showed significantly lower levels of Lactobacilli. The number of Lactobacilli were found to be positively correlated to the glucose concentration in saliva. They found no difference in the caries incidence between healthy and diabetic children. The authors attributed their findings to the dietary treatment of diabetic children which gave rise to a reduction in the Lactobacilli count in saliva but did not affect the Streptococcus mutans count (Twetman et al., 1989; Twetman et al., 1992). Other studies have reported no difference in the Streptococcus mutans and Lactobacillus counts or in the caries incidence between healthy and diabetic children (Swanljung et al., 1992, Iughetti et al., 1999; Siudikiene et al., 2008). Interestingly, in 2002 in a 3 year follow-up study Twetman et al. reported that poor oral hygiene, previous caries experience and high levels of salivary Lactobacilli were the significant single factors for the development of caries in type 1 diabetics. The findings were rationalised by the theory that excess glucose in saliva and gingival crevicular fluid enters into the oral cavity in metabolically poorly controlled cases (Reuterving et al., 1987). This increased the concentration of sugar in the biofilm thus enhancing plaque growth in general and creating ecological conditions favouring aciduric bacteria such as Lactobacilli (van Houte, 1994). Twetman suggested that this finding may reflect the less restricted diet in today's diabetics (Twetman et al., 2002). Bolgül showed an increase in the levels of salivary Streptococcus mutans and Lactobacilli in poorly controlled diabetic children compared to well or moderately controlled type 1 diabetics (Bolgül et al., 2004). Another study demonstrated that the inheritance of some types of HLA-DR4, that are most prevalent in type 1 diabetics, was related to low salivary IgA activity against the streptococcus mutans. This may possibly be caused by a weak bond between these alleles and the antigenic peptides of Streptococcus mutans and may result in increased colonization (Wallengren et al., 2005).

Thus it can be concluded, that studies on the levels of these bacteria in the saliva of diabetic children are inconclusive, reporting decreased, increased or similar levels in comparison to healthy children.

Siudikiene et al. did, however, show that although there were no differences in the bacterial counts of diabetic and non-diabetic children, within the diabetic group there was an inverse correlation of high salivary microbial counts and the level of metabolic control of type 1 diabetes. Poorly controlled diabetics had significantly higher counts of Streptococcus mutans and yeasts, as well as a tendency for higher counts of Lactobacilli, as compared to the well-to-moderately controlled diabetics (Siudikiene et al., 2006). This was also supported by Syrjälä et al. (2003) who showed that among the diabetics with HbA1c≥ 8.5, the presence of dental caries was more distinctly associated with higher levels of Streptococcus mutans and Lactobacilli compared to those with HbA1c ≤8.5. Similarly other studies have also deduced that poor glycemic control strengthens the positive association of dental caries with Streptococcus mutans and Lactobacilli (Karjalainen et al., 1997; Twetman et al., 1992; Twetman et al., 2002).

c. Dental plaque

Dental plaque harbours bacteria that are essential for the development of caries and periodontal disease. Thus the oral hygiene levels are positively related to the levels of incidence of dental caries and periodontal disease in a given population. Older as well as more recent studies have revealed that diabetics had higher plaque indices than the healthy controls (Faulconbridge et al., 1981; Goteiner et al., 1986). Siudikiene et al. have shown that in spite of similar oral hygiene habits, the children with type 1 diabetes were more susceptible to the accumulation of calculus than the healthy controls. Furthermore, the oral hygiene index was found to be significantly higher in children with poor metabolic control of diabetes compared to those with a well to moderate control of the disease (Siudikiene et al., 2005). In a 2 year longitudinal study, the same authors showed that type 1 diabetics demonstrated significantly higher dental plaque levels than the controls. However, the diabetics and their non-diabetic

matched controls did not differ with respect to changes in dental plaque scores over the two year period (Siudikiene et al., 2008). Similar results were found by Lalla et al. (Lalla et al., 2006a). On the other hand, Saes Busato et al. (2010) demonstrated no effect of the metabolic control on the oral health of adolescents with type 1 diabetes.

2.4.1 Type 1 diabetes and dietary factors

Diet is another factor that executes an important function in the development of dental caries. Concurrently, diet plays a significant role in the management of diabetes. Thus common grounds have lead diet to be widely investigated in diabetics in order to discover the implications of each factor in this triad (diabetes, diet and dental caries).

Traditionally, studies focused on diabetics hypothesized that diabetics had a low caries rate due to their sucrose-restricted diet. However, over the years the dietary management of diabetics, especially type 1 diabetics has evolved and their dietary plan does not differentiated much from that of a healthy individual.

Few current studies exhibit improvements in the therapy with insulin and oral anti-diabetics, which have allowed a better control of the disease, without the need for strict dietary restrictions (Tonella et al., 2010). Between 1992 and 1994 Moore et al. surveyed 406 patients with type 1 diabetes regarding their dietary behavior. They found that the records of the diabetics varied extensively from those of the controls. This was attributed to the counseling that the diabetics received to maintain a healthy balanced diet. Dietary recommendations were based on balancing the food intake to daily exercise activities and insulin regimen in order to avoid dramatic variations in daily blood glucose levels and prevent hypoglycemic episodes. The authors found that the diabetics, especially females, had a higher frequency of food intake and snacked more frequently between meals. This potential risk factor for dental caries was not significant in the final regression model even when the gender variable was omitted. Nonetheless, this finding did not indicate a significant association with a history of caries for the entire population. It was concluded that the frequent intake of

carbohydrates (meals and multiple snacks) is likely to be an important risk factor for caries in only a limited number of highly susceptible individuals. Individuals with the most frequent food intakes (6.5/day) were found to have the most severe caries experience. Neither dietary behaviours nor glycemic control were found to contribute to caries in the participants (Moore et al., 2001b).

In 2005, Siudikiene et al. questioned 70 diabetic children and found no major differences between them and non-diabetic children with respect to frequency of meals or consumption of carbohydrates. They found that the diabetic children had more frequent main meals and snacked less than the controls. In addition, the diabetic children drank lesser sweet drinks than the controls.

In another study Ciglar et al. (2002) found that diabetics had a significantly lower daily intake of carbohydrates as compared to the non-diabetics. On the other hand, the diabetics had a considerably higher number of daily meals, intake of dietary fibers, calcium and phosphorus. Additionally, they showed a more frequent presence of low molecular carbohydrates in the daily meals thus aiding the metabolism of cariogenic microorganisms. A significantly higher incidence of caries was found in the diabetics, which the authors ascribed to the more frequent daily intake of low molecular carbohydrates with an improper calcium phosphorus ratio.

2.4.2 Type 1 diabetes and periodontal disease

A general consensus has long been reached that patients with diabetes have an increased risk of developing periodontal disease. It is said to be twice as prevalent in diabetics as in healthy individuals (Grossi, 2001). Most of the studies that support this view have, however, been carried out on patients with type 2 diabetes. Diabetes is considered as a predisposing or modifying factor with regards to the intensity of the host response initiated by a local etiological factor (Carranza & Newman, 1996).

Dental plaque and gingival inflammation

Plaque accumulation and colonization of microorganisms in the periodontal pockets of diabetic patients has been found to be more rapid and to be of a more severe nature than in healthy controls. Furthermore, it has been found that patients with type 1 diabetes develop an earlier and higher inflammatory response to a bacterial challenge than their healthy counterparts (Salvi et al., 2005). Studies carried out on children and adolescents are not as many as those carried out on type 2 diabetes populations, nevertheless, most of them have come to a similar conclusion. Parameters for the analysis of periodontal disease in investigations among children have usually been plaque indices, gingival inflammation and bleeding on probing, as other parameters suggestive of periodontal disease (pocket depth, bone loss) are rare in individuals less than twenty years of age (Karjalainen, 2000). In 1982, Cianciola et al. demonstrated a high incidence of periodontitis in diabetic children, starting at approximately 12 years of age. They reported that 9.8% of the diabetic children showed a generalized periodontitis as compared to only 1.7% of the healthy children (Cianciola et al., 1982). Firatli et al. also reported similar findings in 1996. They measured the plaque index, gingival index, periodontal pocket depths and clinical attachment levels in 77 children and adolescents with diabetes and found the above parameters to be significantly higher than those of the controls. They also found a positive correlation between the duration of diabetes and clinical attachment loss (Firatli et al., 1996). In a 5 year follow-up study, one of the authors in the above study was able to confirm the above results and they concluded “that diabetes modifies the clinical status of the periodontal tissues and increases clinical attachment loss” (Firatli, 1997). A more recent study by Lalla et al. found that diabetes is highly significantly correlated with periodontitis, especially in 12- to 18-year-old children. Children with diabetes had significantly higher plaque and gingival inflammation levels and more number of teeth with evidence of attachment loss than healthy children. The authors concluded that in diabetic children periodontal destruction can start very early in life and become predominant in adolescents (Lalla et al. 2006a). Barnett et al. studied a sample of type 1 diabetics aged between 10 and 18 years and reported no

signs of periodontitis despite many of the subjects in the study having poorly controlled diabetes for a long duration (Barnett et al., 1984).

Several studies have shown a significantly higher gingival index, suggesting increased gingival inflammation in diabetic children and adolescents but no difference in pocket depths/ periodontal status (Pinson et al., 1995; Sandholm et al., 1989b; Novaes et al., 1991). In a group of 50 diabetic children Orbak et al. found gingival inflammation in 69.7% of the 5-9 year-olds, and 83.7% of the 10-14 year-olds. The plaque indices, gingival indices and calculus indices showed an increase with age in the diabetics children, with a significant difference in all the above indices between the diabetic and the control children for the 10-14 year-olds (Orbak et al., 2008).

Goteiner et al. on the other hand, reported gingival inflammation to be equal in diabetic children and adolescents when compared to their controls (Goteiner et al., 1986). In a 3 year longitudinal study, consisting of a group of diabetic children and their healthy siblings, Sbordone et al. found no differences in probing depth, attachment level, sulcus bleeding index or the plaque index (Sbordone et al., 1998).

Therefore, the majority of studies on type 1 diabetic children, based on clinical parameters reveal an increase in gingival inflammation in diabetic children as compared to their controls, signaling an increased susceptibility to the development of periodontal disease later in life. Not all studies have always considered the same clinical parameters (bleeding on probing, plaque accumulation and gingival inflammation) thus making it difficult to actually assess the risk of development of periodontal disease in child diabetic patients.

Earlier studies have focused on the importance of metabolic control for oral health among diabetics. Poor metabolic control has been linked to gingival inflammation and periodontitis (Gusberti et al., 1983; Firatli et al., 1994). At the same time, it has been suggested that an improvement in the periodontal condition in turn has a positive effect on the metabolic control. This opinion is, however, not supported by all studies. Several studies have reported the lack of

an association between gingival inflammation and metabolic control (Pinson et al., 1995; Sandholm et al., 1989b; Sastrowijoto et al., 1989).

Microbial flora

The hypothesis that the composition of plaque differs in diabetic patients, in terms of microbial flora has also been investigated by a number of studies. McNamara et al. examined the microflora of the rat gingival crevice in experimental diabetes model and noted that plaque accumulation was markedly increased in the diabetic rats. A notable change was also found in the gingival microflora which was attributed to diabetes-induced alterations in the sulcular environment (increased level of glucose and urea, and decreased oxygen). Moreover, the microbiological changes were found to precede the development of the periodontal pocket (McNamara et al., 1982). Mashimo et al. investigated the periodontal pockets in children with type 1 diabetes and found an increased prevalence of Capnocytophaga. Actinobacillus actinomycetemcomitans was also detected in some patients. This study, however had no controls (Mashimo et al., 1983). Sandholm et al. also studied the morphotypes of the subgingival microflora in 85 Finnish adolescents with type 1 diabetes. The microflora of the diabetics showed significantly and higher proportions of gram-negative rods, fusiforms, and total gram-negative bacteria. They justified their finding of increased gingival inflammation in this group of patients than in the controls, by the significantly higher proportion of the periodontally pathogenic bacteria (Sandholm et al., 1989a). Other studies have found no significant differences in the microbial species between the diabetics and healthy controls (Sbordone et al., 1995; Sbordone et al., 1998; Christgau et al., 1998). In 2006, Lalla investigated the levels of subgingival plaque bacteria and serum IgG responses in patients with type 1 diabetes and non-diabetic controls with a similar periodontal status. The analysis of 12 bacterial species was done by checkerboard hybridization. The results demonstrated that among the investigated species, only levels of Eubacterium nodatum were found to be higher in diabetic patients, while none of the immunoglobulin G (IgG) titres differed between the groups. The authors concluded that patients with type 1

diabetes and healthy controls showed comparable subgingival infection patterns and serum antibody responses (Lalla et al., 2006b).

Porphyromonas gingivalis is considered as one of the microorganisms responsible for periodontal disease. It has the capacity to invade endothelial cells and is a potent signal for monocyte and macrophage activation. “Thus, once established in the diabetic host, this chronic infection complicates diabetes control and increases the occurrence and severity of microvascular and macrovascular complications” (Grossi, 2001). In 1996, Smith et al. investigated the presence of three periodontal pathogens viz. Porphyromonas gingivalis, Bacteroides forsythus, and Actinobacillus actinomycetemcomitans and serum antibody titers to these bacteria in patients with type 1 diabetes. Porphyromonas gingivalis was detected in 12% of the patients, Bacteroides forsythus in 50% of patients while A. actinomycetemcomitans remained undetected. Positive associations were found between the presence of Bacteroides forsythus and gingival index, probing depth, and attachment level. The study further revealed a depressed humoral immune response among diabetics with lower IgG antibody titres against Porphyromonas gingivalis and Bacteroides forsythus in sera of these patients (Smith et al., 1996).

Thorstensson et al. conducted a study on adult type 1 diabetics and found that significantly more diabetics harboured P. gingivalis than the controls. However, the prevalence of P. gingivalis was associated with deepened periodontal pockets among healthy controls but not among the diabetics. The serum antibody titres for most antigens were found to be similar in both the diabetic and non-diabetic group (Thorstensson et al., 1995). Similar findings were supported by another study (Sastrowijoto et al., 1989). Mandell et al. examined one periodontally-healthy and one periodontally-diseased site in each patient, in a sample of poorly controlled type 1 diabetics. They found increased levels of the periodontal pathogens Prevotella intermedia, P. melaninogenica spp., Bacteroides gracilis, Eikenella corrodens, Fusobacterium nucleatum and Campylobacter rectus in the periodontally diseased sites. Sites associated with

deep pockets and attachment loss revealed higher levels of P. intermedia (Mandell et al., 1992).

Studies investigating periodontally pathogenic microorganisms in type 2 diabetics have revealed a significant increase in the frequency of P. gingivalis, Campylobacter spp., T. forsythensis and A. actinomycetemcomitans in the subgingival plaque of diabetics compared with non-diabetics using polymerase chain reaction (Ebersole et al., 2008; Campus et al., 2005). Using PCR and targeting the 16S rRNA gene, Gafan demonstrated that the three periodontal pathogens, viz. P. gingivalis, T. forsythensis and A. actinomycetemcomitans can be detected in the dental plaque of healthy children and of those with gingivitis. Interestingly, they also found that T. forsythensis was associated more frequently with dental plaque at sites with no gingivitis (Gafan et al., 2004). Similar results were observed by Sakai et al. in 2007. They examined 64 children in a 1 year longitudinal study and found that at least one of the putative periodontal pathogens was detected in a high percentage of the children even though the children depicted periodontally healthy conditions (Sakai et al., 2007).

At present, it is difficult to estimate the real effect that diabetes has on the composition of the subgingival plaque (microorganisms). Despite the fact that certain microorganisms are more prevalent in diabetics, whether this is due to direct alterations in the subgingival microenvironment or whether it takes place indirectly by alterations to the host response, remains unclear. The subgingival microenvironment of diabetic patients might be altered as a result of hyperglycaemia, making it more habitable for periodontally pathogenic bacteria. On the other hand, the host response to plaque may be altered in diabetics leading to increased tissue destruction (Ohlrich et al., 2010).

2.5 Phenylketonuria

Phenylketonuria (PKU) is a rare metabolic genetic disorder. It is inherited as an autosomal recessive trait and characterised by a wide range (over 400) of mutations in the gene coding for the hepatic enzyme phenylalanine hydroxylase (PAH). This leads to an absence or deficiency of the enzyme phenylalanine hydroxylase which is essential to metabolise the amino acid phenylalanine (Phe) into the amino acid tyrosine. Clinical manifestations of the disorder are due to toxic accumulation of phenylalanine in the blood and brain.

Generally, PKU is diagnosed in a neonatal screening. However, if undiagnosed at birth, symptoms start developing within a few months. They range from very mild to severe and comprise of gradual developmental delay, stunted growth, microcephaly, seizures, tremors, eczema, vomiting, and a musty odor. Untreated patients, in time, develop intellectual disability, behavioral disorders (hyperactivity) and motor disorders. Three forms of PKU have been described, viz. classical (Phe > 20 mg/dl [1200 µmol/l]), mild (Phe between 10mg/dl [600 µmol/l] and 20 mg/dl [1200 µmol/l]) and mild hyperphenylalaninemia (Phe < 10 mg/dl, [600 µmol/l]).

The classical form is the most common form of PKU and manifests itself with severe symptoms. Classic PKU is caused by a complete or near-complete deficiency of PAH activity. Individuals with untreated classical PKU often present with a musty body odor and dermatological conditions due to the excretion of excessive phenylalanine and its metabolites. A decrease in skin and hair pigmentation might also be observed due to the associated inhibition of tyrosinase. Due to problems in the recycling of tetrahydrobiopterin (BH4), the cofactor in the phenylalanine, tyrosine, and tryptophan hydroxylation reactions, involved in catecholamine, serotonin, and nitric oxide biosynthesis, patients may also show a decreased myelin formation and dopamine, norepinephrine, and serotonin production. Additional problems, like exaggerated deep tendon reflexes, tremor, and paraplegia or hemiplegia, can arise later in life (Williams, 1998; Pérez-Dueñas et al. 2005).

2.5.1 Prevalence

The prevalence of PKU is estimated to be 1 in 10,000 live births in Europe (Steinfeld et al., 2004). The gene mutation frequency varies between different ethnic groups, thus showing a higher rate in some countries like Ireland and Italy. Prevalence is reported to be particularly high in Turkey with 1 in 4,000 live births (Stuhrmann et al., 1989). In Germany an estimated 60 newborns are diagnosed with PKU every year. PKU is found to be rarer in Finland, Africa and Japan (Mitchell and Scriver, 2000).

2.5.2 Treatment and dietary considerations

There is no remedy for PKU. The objective in the management of PKU is to prevent developmental delay and assist in normal growth of the patient. This is accomplished by maintaining lower blood phenylalanine concentrations to the recommended therapeutic levels. A low-protein diet in combination with a protein (amino acids) Phe-free medical formula, which is free from or low in phenylalanine is the mainstay of treatment. The aim is to achieve plasma Phe concentrations of 2-5 mg/dL (120-300 µmol/L) in newborns and young children. Patients tolerate less than 250-350 mg of dietary phenylalanine per day to keep plasma concentration of Phe at a safe level of no more than 5 mg/dL (300 µmol/L). Controversy, however, remains over the plasma Phe concentration to be attained for individuals older than twelve years of age. The overall consensus is that the closer the Phe concentration is to the recommended normal value, the better is the patient's general state of well-being. A diet which is low in phenylalanine should be initiated as soon as possible after birth and continued at least into adolescence and when necessary, for life. Children under two years of age should maintain a total amino acid intake of at least 3 g/kg/day including 25 mg tyrosine/kg/day. In order to minimize fluctuations in blood amino acid concentrations, the consumption of Phe-free medical formula should be spread out equally over 24 hours. An adjuvant therapy with 6R-BH4 stereoisomer may benefit a large percentage of patients with PKU. In addition to monitoring of the plasma Phe and Tyr concentrations, diet and cognitive

development of the patient, must be evaluated at regular intervals (Mitchell & Scriver, 2000). Guidance regarding the use, dosage, and distribution of dosage of the protein substitute over a 24-hour period is ambiguous, with a discrepancy in recommendations between different treatment centres (Yi & Singh, 2008).

Medical Foods

As mentioned above, the treatment of PKU entails a strict diet which is low in proteins and a Phe-free medical formula that substitutes the proteins (essential amino acids) required by the patient for normal cognitive and physical development. 'Medical foods' is a term used to describe the Phe-free medical formula and foods modified to be low in protein. In addition to the essential amino acids the medical formula provides tyrosine, vitamins, minerals and trace elements that a person without PKU would get from their normal diet. The medical foods and more importantly the Phe-free formula is generally prescribed by the physician. It is critical for patients with PKU to take the prescribed amount of medical formula each day. The nutritional needs of each patient are assessed by the dietitian and accordingly the formula is prescribed. Formulas are available in a variety of forms and flavours, as a powdered drink mix or as a ready-made drink in different flavours, bars and tablets to suit diverse lifestyles and preferences. Formula is usually consumed approximately three to four times a day, depending on individual requirements.

Additionally, low protein foods are modified by the manufacturer such that they supply no more than one gram of protein per serving. They provide the additional calories required by the patient without supplying additional phenylalanine containing protein. In this manner, catabolism which in itself can cause Phe levels to rise may be prevented.

A substantial amount of the protein required by the PKU patients is derived from the medical formula, the rest, which includes the essential amount of Phe the body needs for functioning and development, is derived from food. The PKU diet primarily comprises of low protein foods such as fruits, vegetables, modified low protein foods, fats and sugars.

Foods, high in protein such as red meat, chicken, fish, eggs, milk, yogurt, cheese, nuts, soybeans and beans are excluded from a PKU diet, except when the dietary restriction required is minimal. Foods such as regular pasta, bread, rice and starchy vegetables (potatoes, peas, corn etc.) are also meant to be consumed in a very limited amount. Unlimited quantities of low protein foods should also be avoided. Fatty foods and foods with almost no proteins, such as jams and conserves and sweets free from milk and gelatin need not be restricted.

Aspartame

Aspartame avails a special mention in the diet of a patient suffering from PKU. It is an artificial sweetener, used often in several 'sugar-free' foods, beverages and medications, that contains phenylalanine. Individuals with PKU should avoid eating or drinking any food or drinks containing aspartame. Therefore, generally most products containing aspartame are labeled with a warning: 'Contains Phenylalanine'.

2.5.3 PKU und oral health

It has been suggested that patients with PKU are more likely to have dental caries and or dental erosion as their diets tend to include more sugary foods and acidic drinks. As mentioned above, children with PKU derive less protein from foods; hence it is necessary for them to consume more carbohydrates and fats to supplement their energy. For the same reasons, they may snack more often over the course of the day. Furthermore, frequent ingestion of the Phe-free medical formulas, which are sweetened and acidic, is detrimental for oral health.

In 1999, Kilpatrick et al. evaluated the oral health of forty children with PKU and the erosive potential of 5 amino acid supplements commonly prescribed in their management. They found no significant difference in the level of dental caries between the PKU children and the healthy children. The PKU children did, however, show more signs of tooth wear. Supporting this finding, the study did reveal that the titratable acidity of the flavoured supplements was significantly

higher than the control drinks (Kilpatrick et al., 1999). Another case report published in 1992, describing the management of a child with PKU, tried to provide a causal relationship between the chronic dietary acid load with dental caries (Manz & Schmidt, 1992).

A search for studies and literature on children with PKU and dental health is relatively fruitless. Very few studies have been conducted on the subject. Less than a handful of studies, conducted in Europe in the early 70's and 80's have focused on the oral health of children with PKU. For the purpose of the present thesis these studies are, however, of little or no reference value as they have been published in the local languages (Stavljenić et al., 1987; Oulis & Kostopoulou, 1984; Velková & Podhradská 1976; Velková & Podhradská 1981; Ogresta & Zaninović, 1975).

2.6 Detection of microorganisms using Polymerase Chain Reaction

Traditionally, conventional culture methods have been widely used in studies to distinguish the composition of oral microflora and are still considered the gold standard when determining the utility of a new microbial diagnostic method in oral microbiology. It is the only method able to properly describe new species and to assess the antibiotic susceptibility of the grown bacteria. The conventional culture methods do, however, have some drawbacks. Only viable bacteria may be grown thus necessitating strict sampling and transport conditions. Furthermore, culture of certain putative periodontal pathogens can prove to be rather problematic. The sensitivity of this method can be slightly low. Therefore, low numbers of a specific pathogen in a sample will remain undetected and could falsify results. An additional drawback is that in this day and age, culture methods are proving to be relatively time-consuming (Sanz et al., 2004).

Over almost two decades Polymerase Chain Reaction has established itself as a reliable DNA-based technique for detection of human oral microbial

pathogens. It has been found to be a rapid, accurate, highly sensitive and specific method for the detection of periodontal pathogens as well as bacteria associated with caries (Loesche, 1992; Lyons et al., 2000; Boutaga et al., 2006; Rupf et al., 1999).

The advancements in molecular biology, designed for the detection of microorganisms, have provided an improvement in the knowledge of microbial genetics and has further boosted the development of enhanced diagnostic techniques (Holt & Progulske, 1988; Gibbs, 1990; Saiki et al., 1988). Molecular biology techniques are aimed at the analysis of DNA, RNA or the protein structure or function (Dawson et al., 1996). The genetic material of a microorganism constitutes transferring, ribosomal and messenger RNA and chromosomal DNA. The principle of molecular biological diagnostic tests is based on the recognition of specific (complementary) bacterial DNA sequences from target microorganisms by specific DNA fragments. Therefore, it is essential to extract bacterial DNA from the sample and amplify the specific DNA sequence of the target bacteria. Different chemical, enzymatic or physical methods are implemented to extract sufficient quantity of DNA for the subsequent analysis by polymerase chain reaction (PCR). Organic chemicals or detergents, such as hexadecyltrimethyl ammonium bromide or guanidine hydrochloride are used for the lysis of the cells and precipitation of DNA is carried out with isopropanol or ethanol (Smith et al., 1989). In another method, lysozyme, an enzyme, is used to split the bacterial wall and the proteinic components of the cell are destroyed with proteinase K. Heat is often used for the disruption of the cell and denaturation of the proteins. Consequently, the separation and purification of DNA is carried out using centrifugation and chromatographic columns. Generally, extraction of DNA for periodontal testing purposes is done using proteinase K or boiling and centrifugation (Ting & Slots, 1997; Umeda et al., 1998). Following the extraction and purification of DNA from the sample polymerase chain reaction is used to specifically detect and quantify the target pathogens.

PCR is an extremely sensitive diagnostic technique, capable of detecting even one copy of the searched DNA fragment (Greenstein, 1988). However, just as any other diagnostic technique it also has some limitations. A main drawback of PCR is its vulnerability to contamination. The specificity of the polymerase chain reaction depends on several complex, interrelated factors, such as oligonucleotide primer size, annealing temperature and buffer salt concentration. A small change or miscalculation of any of the ingredients can lead to major errors in the result. Additionally, problems may arise when studying small quantities of DNA, since the constituents of the reaction (oligonucleotide primers, dNTPs, Taq polymerase) may be exhausted before sufficient target is produced. On the other hand, PCR provides many advantages in the diagnosis of oral microorganisms. Compared to conventional lengthy and time consuming techniques, analysis of a great number of samples can be carried out relatively easily and efficiently.

3 Aims and objectives of the study

The aim of this study was to investigate the oral parameters that influence the caries risk and risk of developing periodontal disease in children with PKU and type 1 diabetes. The parameters to be assessed were the dental and oral hygiene status and oral microorganisms in children with diabetes, PKU and in healthy children.

The null hypothesis of our study is that the children and adolescents suffering from phenylketonuria do not possess a higher caries rate and more signs of periodontal disease than their healthy or diabetic counterparts. On the other hand, diabetic children do not have a lower caries rate and a higher risk of developing periodontal disease.

The alternative hypothesis of our study is that the children suffering from phenylketonuria possess a higher caries rate and show lesser signs of periodontal disease than their healthy or diabetic counterparts. On the other hand, diabetic children have a lower caries rate and a higher risk of developing periodontal disease.

4 Material and Methods

This study, conducted at the Heinrich-Heine-University, Düsseldorf was a cross-sectional study and involved the examination of patients from 3 groups. The study was carried out from September 2005 till November 2006.

Children suffering from PKU (Group A) und type 1 diabetes (Group B) were recruited from the Paediatric Department of the Children's Hospital at the University Hospital in Düsseldorf. The control group (Group C) consisted of healthy children who were recruited from the Department of Operative Dentistry, Periodontology and Endodontology and from the Department of Orthodontics, University Hospital, Düsseldorf. Children between the ages 3 and 18 years were recruited for the study. The total sample consisted of 238 children. Both Group B and C comprised of 100 children and Group A had 38 children. The patients were matched for gender and age.

Parents of all the children gave their informed and written consent before participating in the study. This study was approved by the Institutional Human Subjects Ethics Committee of the Heinrich-Heine-University, Düsseldorf (Study number: 2644).

Inclusion Criteria:

- Children diagnosed with either type 1 diabetes or phenylketonuria (group A & B)
- Aged between 3 and 18 years
- Informed consent given by the parents

Exclusion criteria:

- Intake of antibiotics 2 weeks prior to the clinical examination
- Systemic disease (group C)
- An uncooperative child

4.1 The Questionnaire

Information regarding economic and social status, oral hygiene habits and fluoride intake was collected with the help of a questionnaire (see Appendix). The questionnaire was filled out by one of the parents or guardian in the waiting room or while the child was being examined. The part of the questionnaire collecting information on the socioeconomic status included questions regarding the educational and professional status of both parents. Social status scores were calculated from each of the three components. The child's social status was defined by computing a total score for each parent. The higher of the two scores was used. In the case of the parents being separated, the score of the main care-provider was employed. Accordingly, the categories of social status were defined as lower, middle and upper (Winkler& Stolzenberg, 1999).

4.2 Clinical Examination

All the participants were examined by a single dentist. Examination of the patients was done under adequate lighting conditions, i.e. using a patient examination light when the patient was not examined in a dental chair (at the children's hospital). All the healthy control patients (group C) were examined in a dental chair at the dental hospital. Two mirrors and a William's probe were used for the dental examination. The dental status was assessed by the DMFS Index and the oral hygiene and gingival health was evaluated by employing the plaque index by Silness & Löe and the Papillary Bleeding Index (PBI), respectively.

4.2.1 DMFS/ dmfs Index

The DMFS Index (decayed, missing, filled surfaces) was used for the secondary (permanent teeth) and the dmfs Index for the primary dentition. The DMFS numerically expresses the prevalence of dental caries in an individual by calculating the number of decayed (D), missing (M), filled (F) teeth and surfaces

(S) of the teeth in that individual. The index helps to provide an assessment of how many teeth in an individual have been affected by dental caries, needed to be extracted due to caries and how many surfaces of the teeth have been needed to be filled due to caries. The index can be calculated for either twenty eight teeth or thirty two teeth, including the wisdom teeth. If a tooth shows both a carious lesion and a filling it is calculated as decayed. The sum of all four figures forms the DMFS value. The anterior teeth have four surfaces each and the posterior teeth have five surfaces each. Therefore, the maximum score, excluding the 3rd permanent molar, for the DMFS index can be 128 for twenty eight teeth. In a similar manner the dmfs index is calculated for the primary teeth. In this case, the 'm' is for teeth that are missing or have been extracted due to caries and not teeth that have been exfoliated normally (WHO, 1997). So as to ease the calculation of the index during the analysis, the teeth or surfaces were coded as follows:

0 = healthy

1 = initial caries

2 = carious

3 = fissure sealant

4 = filled and caries-free

5 = filled and primary carious lesion

6 = filled and secondary carious lesion

7 = missing

8 = unerupted

9 = cannot be assessed

4.2.2 Papillary Bleeding Index (PBI)

The Papillary Bleeding Index was first introduced by Saxer and Muhlemann in 1975. Bleeding is checked by inserting a periodontal probe into the gingival sulcus at the base of the papilla on the mesial side, and then moved coronally to the tip of the papilla. This is then immediately repeated on the distal side of the papilla. The intensity of any bleeding is recorded as:

0 No bleeding

1 A single discreet bleeding point

2 Several isolated bleeding points or a single line of blood appear

3 The interdental triangle fills with blood shortly after probing

4 Profuse bleeding occurs after probing; blood flows immediately into the marginal sulcus.

4.2.3 Silness & Löe Index

Plaque deposits were assessed using the Silness & Löe Index, which is based on recording both soft debris and mineralised deposits on the teeth. Missing teeth are not substituted.

The following scores are given from 0 to 3:

0 No plaque

1 A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only using the probe on the tooth surface.

2 Moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.

3 Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

The index score is achieved by calculating the sum of all the individual score and dividing it by the number of teeth examined.

Metabolic control of diabetes:

The level of metabolic control of diabetes for the children in group B was determined by the glycosylated haemoglobin value HbA1c. This reflects the levels of glycaemia over the preceeding 6-12 weeks. According to the guidelines for the management of type 1 diabetes, diabetics with HbA1c < 9% are considered as well to moderately controlled patients and HbA1c ≥ 9% as poorly controlled (Global IDF/ISPAD Guideline for Diabetes in Childhood and Adolescence, 2011)

4.3 Microbiological sample collection

For the microbiological examination of the bacteria, a sample was collected on a sterile paper point (ISO 35) from the cervical region and gingival sulcus of the first deciduous molar or from the first permanent molar in the lower right quadrant. In case the above mentioned teeth were missing, the sample was taken from the gingival sulcus of the first deciduous molar or from the first permanent molar in the lower left quadrant or the upper right quadrant.

The paper point was scraped along the cervical region of the tooth crown, inserted into the gingival sulcus for a period of 30 seconds and then transferred to a 1.5 ml microfuge tube (Eppendorf, Hamburg, Germany). This tube was transported on ice to the laboratory, and frozen immediately, initially at -18° C and then at -80° C, until later analysis.

4.4 Laboratory analysis of the bacteria- Materials

Quantitative real-time Polymerase Chain Reaction (PCR) was used for the detection of the following bacteria:

1. Lactobacillus species (Lac)
2. Lactobacillus casei (Lca)
3. Streptococcus mutans (Sm)
4. Aggregatibacter actinomycetemcomitans (Aa)
5. Porphyromonas gingivalis (Pg)
6. Tannerella forsythensis (Tf)

4.4.1 Chemicals

The chemicals used in the laboratory were procured from the following companies:

Gibco BRL	Karlsruhe, Germany
Merck	Darmstadt, Germany
Sigma-Aldrich	Seelze, Germany
Biozym	Hessisch Oldendorf, Germany

4.4.2 Primers and Probes

Primers and probes (Table 1) used for the PCR were synthesised by:

Metabion	Martinsried, Germany
Eurogentec S.A.	Seraing, Belgium (probe for Tf)

Species	Primer/Probe	Sequence (5'-3')	Size	Literature
Sm	Smu-AJ243965-F	TGG GAC GCA AGG GAA CA	17 nt	Price et al.2007
	Smu-AJ243965-R	CCC GTT CGC GAC TCA AGA	18 nt	
	Smu-AJ243965-T(HEX-BHQ1)	CTT GCA CAC CGT GTT T	16 nt	
Lac	Lacto- F2	TGG AAA CAG ATG CTA ATA CCG	21 nt	Byun et al.,2004
	Lacto-R2	CGT CCA TTG TGG TAG ATT CCC T	22 nt	
	Lacto-S(FAM-BHQ1)	CTG AGA CAC GGC CCA WAC TCC TAC GG	26 nt	
Lca	Lcase-62F	TGC TTG CAC CGA GAT TCA AC	20 nt	Self-designed
	Lcase-162R	TTA GCA TCT GTT TCC AAA TGT TAT CC	26 nt	
	Lcas95T(HEX-BHQ1)	CGG ACG GGT GAG TAA CAC GTG GGT AAC	27 nt	
Aa	Aa-U51862-F	ACG CAG ACG ATT GAC TGA ATT TAA	24 nt	Morillo et al. 2004
	Aa-U51862-R	GAT CTT CAC AGC TAT ATG GCA GCT A	25 nt	
	Aa-U51862-S(FAM-BHQ1)	TCA CCC TTC TAC CGT TGC CAT GGG	24 nt	
Pg	Pg-D64081-F	CCT ACG TGT ACG GAC AGA GCT ATA	24 nt	Morillo et al. 2004
	Pg-D64081-R	AGG ATC GCT CAG CGT AGC GAT T	22 nt	
	Pg-D64081-S(TEXRED-BHQ2)	TCG CCC GGG AAG AAC TTG TCT TCA	24 nt	
Tf	Tf-AF054892-F	TCC CAA AGA CGC GGA TAT CA	20 nt	Morillo et al. 2004
	Tf-AF054892-R	ACG GTC GCG ATG TCA TTG T	19 nt	
	Tf-AF054892-S(FAM-BHQ1)	TCG CGA CGT GAA ATG GTA TTC CTC	24 nt	

Table 1: Primers and probes for the investigated bacteria

4.4.3 Master Mix

- *qPCR Mastermix No ROX (Cat.-No: RT-QP2X-03NR)*

  Eurogentec Seraing, Belgium

4.4.4 Kits

- *Rapid DNA Ligation Kit*

  Roche Diagnostics Mannheim, Germany

- *High Pure PCR Product Purification Kit*

  Roche Diagnostics Mannheim,Germany

- *QiAamp DNA-Mini Kit*

  Qiagen Hilden, Deutschland

- *Gentra Puregene Kit*

  Qiagen Hilden, Germany

4.4.5 Enzymes

- *Proteinase K*

  Sigma-Aldrich Seelze, Germany

- *Taq DNA Polymerase*

  Invitrogen Carlsbad, USA

- *Restriction endonucleases Sph I und Sal I*

  Amersham Buckinghamshire, UK

4.4.6 Bacterial Strains

The bacterial strains used in this study were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany), American Type Culture Collection (ATCC) (LGC Standards, Wesel, Germany) and from the reference cultures of the Institute for Medical Microbiology and Hospital Hygiene, Heinrich-Heine University, Düsseldorf. They were used to isolate genomic DNA for use in the Real-Time PCR and to set up the PCR Standards.

Species	
E.coli	DH5α
Streptococcus mutans	Reference culture
Lactobacillus casei	Reference culture
Aggregatibacter actinomycetemcomitans	DSMZ
Prophyromonas gingivalis	DSMZ
Tannerella forsythensis	ATCC

Table 2: Procurement of bacterial strains

4.4.7 Cloning Vector

Plasmid	Size (kb)	Resistance gene	Character	Company
pGEM-T	3	Amp	LacZ' Gene, SP6- und T7 Promotor, f1-ori	Promega, Mannheim, Germany

Table 3: Cloning Vector

4.4.8 Equipment

- *iCycler iQ5*

  BioRad Munich, Germany

- *T-Gradient PCR thermalcycler*

  Whatman Biometra Göttingen, Germany

4.5 Laboratory analysis of the bacteria - Methods

4.5.1 DNA Extraction

a. Extraction of genomic DNA from patient samples

1. The paper point sample was inserted into a microfuge tube (Eppendorf, Hamburg, Germany) with 70 µl 10M Tris HCl pH 8.0.
2. 140 µl Proteinase K was added to the microfuge tube and vortexed for 30 secs.
3. The microfuge tube was placed in a thermo-mixer at 56° C, set at 600 rpm for 1 hour. After 30 mins, the tube was removed and vortexed for 30 secs and reinserted.

4. The microfuge tube was then boiled for 30 mins, cooled, vortexed for 30 secs and finally centrifuged for 2 mins.
5. The resulting eluate was pipetted into a new microfuge tube and frozen at -20° C until further use. The paper point was discarded.

b. Extraction of genomic DNA from bacterial cultures

The QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's instruction to extract the DNA from the bacterial cultures in the following manner:

1. 2 ml of the bacterial culture was centrifuged for 10 mins (13,000 x g).
2. The resulting bacterial sediment was re-suspended in 200 µl PBS.
3. 20 µl Proteinase K (10 mg/ml) and 200 µl buffer AL was added to the suspension, mixed by vortexing for 15 secs, and then incubated at 56 °C in the thermal-block for 10 mins so that the sample was completely lysed.
4. 200 µl Ethanol 96 (w/v) % was added to the sample, mixed by pulse-vortexing for 15 secs. The microfuge tube was centrifuged briefly to remove drops from the inside of the lid.
5. The mixture was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube). This was then centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp mini spin column has a silica membrane which is capable of binding the DNA while the column is centrifuged.
6. In order to purify the eluted DNA, the silica membrane was washed with 500 µl AW1 Buffer and AW2 Buffer.
7. DNA was eluted from the membrane by pipetting 200 µl AE Buffer on the silica membrane.
8. The mini spin column was placed in an elution jar and incubated for 5 mins at room temperature before being finally centrifuged at 6000 x g for a minute.
9. The eluded DNA obtained by the above method was stored at -20°C until further use.

c. Isolation of Plasmid DNA from E.Coli

1. E. coli culture was inoculated with 3 ml of sterile LB-Amp Medium (1% trypton, 0.5% yeast, 0.5% NaCl, 100 µg/µl Ampicillin, pH 7.5) and incubated overnight at 37°C.
2. 1.5 ml of this culture was transfered to a microcentrifuge tube and centrifuged at 13000 x g for 2 mins.
3. After dumping the supernatant 300 µl of Solution 1 (50 mM Tris/HCl pH 8.0, 10 mM EDTA) was added to the cell pellet, the cells were resuspended and 12 µl RNAse 1 solution was added to them.
4. 300 µl of solution 2 (0.2 M NaOH, 1% SDS) was added. This solution ruptures the cells.
5. 300 µl of solution 3 (2.55 M potassium acetate, pH 4.8) was added, the tubes capped and shaken vigorously. This step precipitates the chromosomal DNA and the proteins.
6. In order to separate the plasmid DNA from the chromosomal DNA and the cellular debris in the pellet the tube was centrifuge at 13000 x g for 20 mins.
7. 800 µl of the white precipitate was mixed with 640 µl isopropanol and centrifuged at 13000 x g for 15 mins. Isopropanol precipitates the nucleic acids.
8. 500 µl of ice-cold 70% ethanol was added and the tube centrifuged once again.
9. The tube was allowed to dry for approx. 5 mins and the resulting DNA stored in 30 µl 10 mM Tris/ HCl, pH 8.0 at - 20°C.

The concentration of the plasmid DNA was determined with the help of a spectrophotometer at 260 nm. The concentrations of the DNA ranged from 6.5 - 14.5 ng/ µl.

4.5.2 Cloning

a. Ligation

Approximately 2.5 ng amplicon was ligated into 0.25 ng pGEM-T-vector using the Rapid DNA Ligation Kit (Roche Diagnostics, Mannheim, Germany). 1 µl DNA 5X elution buffer, 5 µl T4 DNA 2X ligation buffer and 0.5 µl T4 DNA ligase (5 U/ µl) were added to the vector and amplicon. This was carefully mixed with a pipette and then incubated at room temperature for 30 mins.

b. Bacterial Transformation

1. 100ul of competent E. coli – DH5α cells were pipetted into the above ligated vector. The tube was placed on ice for 30 min.
2. The tube was then kept in a waterbath at 42^{o}C for exactly 2 mins and immediately returned to ice for 1-2 mins.
3. 800 µl SOC medium(2% Trypton, 0.5% yeast, 0.5% NaCl, pH 7.0, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) was added to the transformation mixture. This was incubated at 37°C for 1 hour with shaking.
4. 1/10 and 9/10 of the mixture was spread on LB –Amp plates (1% Trypton, 0.5% yeast, 0.5% NaCl, 1.5% agar, 100 µg ampicillin, pH 7.5) and placed overnight in the incubator at 37°C .
5. 4 clones were picked from each plate and cultivated in LB-Amp medium (1% Trypton, 0.5% yeast, 0.5% NaCl, 1.5% agar, 100 µg/ µl ampicillin, pH 7.5).
6. The DNA was extracted from the bacteria as described earlier.

c. DNA restriction

4 µl of restriction enzymes *Sph I* and *Sal I,* 84 µl water in 48 µl OPA buffer were added to 5 µl of plasmid DNA (approximately 2.5 ng DNA) and incubated at

37°C for 1 hour. The cloning process was analyzed by gel electrophoresis. Figure 1 shows the pGEM®-T Vector (Promega, Mannhein, Germany) with single 3´-T overhangs at the insertion site. The polymerases add a single deoxyadenosine to the 3´-ends of the amplified fragments. The restriction enzymes *Sph I* and *Sal I* cleave the insert clones at the respective cloning sites. Figure 2 shows the respective cleavage sites.

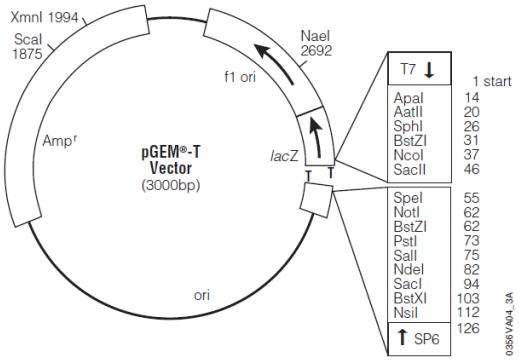


Figure 1: pGEM®-T Vector Map and sequence reference points (Promega /t-vectors)

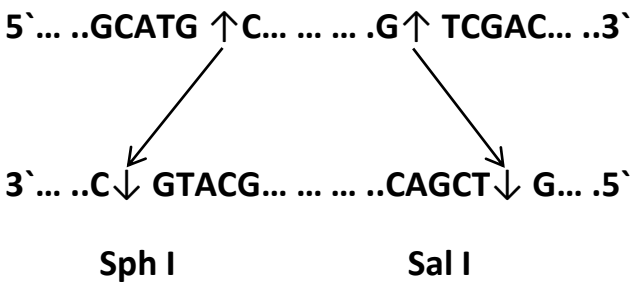


Figure 2: Cleavage site on the restriction endonucleases *Sph I* and *Sal I*

d. Gel electrophoresis analysis of DNA

Gel electrophoresis was used to analyse the restricted or amplified DNA. Depending on the size of the DNA fragment 1.5- 3% agarose gel was used for this purpose. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations that of larger DNAs. 0.5x TBE was

used as the electrophoresis buffer (90mM Tris, 90mM Borate, 0.5 mM EDTA, pH 8.0). DNA samples with loading buffer (0.25% Bromophenol blue, 0.25 Xylene cyanol, 15% Ficoll) were pipetted into the sample well and a current of 50 mA was applied. As a result of the current, the DNA strands migrate resulting in bands of varying thickness. The gel was incubated in 0.1% ethidium bromide solution in order to visualize the DNA bands. The 1-kb MassRuler DNA Ladder (Fermentas, St. Leon-Rot, Germany) was used as the reference to estimate the mass of the bands. The DNA bands were photographed under UV light.

e. Bacterial storage

800 µl of each bacterial culture was stored in LB-Amp medium with 200 µl glycerine (20 % concentration). These cultures were then stored at - 20°C until further use.

f. Determination of the DNA concentration

Spectrophotometry was employed to determine the concentration of DNA. It works by the principle of measuring the amount of ultraviolet irradiation absorbed by the bases. 5 µl of the sample was added to 100 µl of water in the quartz cuvette of the spectrophotometer.

Readings were taken at wavelengths of 260 nm and 280 nm. 260 nm is the absorption maximum for DNA and 280 nm is the absorption maximum for proteins. Therefore, the reading at 280 nm gives the amount of protein in the sample and the reading at 260 nm allows calculation of the concentration of nucleic acid in the sample.

1 O.D. (optical density) at 260 nm for double-stranded DNA = 50 ng/ul of DNA

Pure preparations of DNA have OD260/OD280 values of 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantification of the amount of nucleic acid will not be possible.

The DNA concentration was determined as follows:

OD260 value/ OD280 value x dilution factor

The concentrations of the DNA (C) are shown in the table below:

Plasmid	C (ng/µl)	Molecules/µl
Lca	95	2.8×10^{10}
Sm	74	2.2×10^{10}
Aa	161	4.8×10^{10}
Pg	175	5.2×10^{10}
Tf	67	2.0×10^{10}
Lac	80	2.26×10^{10}

Table 4: DNA concentrations of the investigated bacteria

4.5.3 Polymerase chain reaction (PCR)

The PCR starts with denaturation of the target DNA strand (separation into a single strand). For this purpose the DNA is heated for 1 to several mins to 94° to 96° C. Following this, the temperature is lowered to approximately 50° C in order to allow the forward and reverse primers to anneal to their complementary sequences. The primers are designed to complement the DNA region to be amplified. Subsequently, the temperature is raised to about 72° C. This allows the Taq polymerase to attach to each priming site and produce a new DNA strand. This is the end of the first cycle. The above mentioned steps of denaturation, annealing and extension or elongation are repeated 40 times.

a. Conventional PCR

A T- Gradient thermalcycler (Whatman Biometra, Göttingen, Germany) was used to perform the conventional PCR. The PCR mix consisted of the following:

Template	**0.5 µl DNA solution (ca. 2.5 ng DNA)**
Buffer	5 µl 10 X PE Buffer (10 mM Tris/ HCl, pH 8.3, 50 mM KCl, 1.5mM $MgCl_2$)
Deoxynucleoside triphosphate	8 µl NTP- Mix (each 1.3 mM GTP, CTP, ATP, TTP)
Primer	Each 0.5 µl Primer (0.1 mM)
Aqua dest.	34.5 µl
Taq DNA polymerase	1 µl Taq polymerase

Table 5: Conventional PCR Mix

The PCR run was as follows:

	PCR step	Temperature	Time
1.	Initialization	95 °C	5 min
2.	Denaturation	95 °C	1 min
3.	Annealing	52 °C	1 min
4.	Elongation	72 °C	1 min

Table 6: Conventional PCR Run

A series of 40 cycles were repeated for Steps 2-4. The analysis of the PCR was done with gel electrophoresis as described above.

b. Real-Time PCR

Real- time PCR enable one to measure the amount of PCR product at any point of time. A cycle threshold (C_t) is defined during the exponential phase of the PCR. This reflects the instant at which the fluorescence signal generated from a sample is significantly greater than background fluorescence. In other words, this is the point at which sufficient number of amplicons have accumulated during the reaction. These C_t values are directly proportional to the amount of starting template and represent the basis for calculating the DNA copy numbers. Accurate quantitative results of the PCR product at specific cycle numbers can be achieved by this method (Dorak, 2007a). In a real time PCR, reporter molecules which are fluorescent dyes (SYBR Green) or sequence – specific probes (TaqMan Probes) labeled with both a reporter fluorescent dye and a quencher dye are bound to the double stranded DNA. These reporter molecules produce fluorescence which increases with each amplification cycle due to the accumulation of the PCR product. This fluorescence is detected and

monitored, thus enabling the quantification of even a relatively small amount of PCR product.

TaqMan Probes:

TaqMan probes utilize the 5' exonuclease activity of the enzyme Taq Polymerase for measuring the amount of target sequences in the samples. These probes are oligonucleotides, with a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end, are added to the PCR mixture. The reporter and the quencher remain in close proximity to each other till the probe is hydrolysed. The fluorescence of the reporter dye is not completely quenched by the close proximity to the quencher thus producing background fluorescence. During PCR, the probe anneals specifically between the forward and reverse primer to a specific part of the PCR product. As extension of the primer is carried on the template to which the TaqMan probe is bound, the probe is cleaved and the reporter molecule pushed away from the quencher. This increases the fluorescence intensity of the reporter dye. This increase in fluorescence is quantitated as the reaction progresses.

The probes used in this study were marked with following reporter-quencher combinations:

GAPDH	FAM-BHQ_1
Lca	HEX- BHQ_1
Sm	HEX- BHQ_1
Aa	FAM-BHQ_1
Pg	Texas Red-BHQ_1
Tf	FAM-BHQ_1
Lac	FAM-BHQ_1

Controls:

PCR is especially extremely sensitive to contamination by unwanted template DNA. This poses a fundamental problem when the amount of template DNA is limited. Practical measures are taken during preparation in the laboratory in order to reduce contamination. However, one of the most significant measures is to include control reactions in each PCR experiment. The negative control reaction included exactly the same components as the experimental tubes and water (H_2O) instead of template DNA (patient sample). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was added as the positive control. GAPDH is an essential enzyme also known as a housekeeping gene. Housekeeping genes are genes that are constitutively expressed because they code for proteins that are continuously required by the cell to perform basic functions necessary for the sustenance or maintenance of the cell. GAPDH plays an important role in glycolysis. If GAPDH is not detected in the patient sample it is an indication that either the amount of sample included was insufficient or that the reaction was being inhibited by a contaminant (Ellerbrock, 2010).

The iCycler IQ5 from Biorad (Munich, Germany) was used to perform the Real-Time PCR. A template, comprising of 9 patient samples, 2 standards and a negative control (H_2O) was developed for the Real-Time PCR run (Figure 3).

	1	2	3	4	5	6	7	8	9	10	11	12
GAPDH	Std. 10^5	Std. 10^2										H_2O
Lac	Std. 10^5	Std. 10^2										H_2O
Sm	Std. 10^5	Std. 10^2										H_2O
Aa	Std. 10^5	Std. 10^2										H_2O
Pg	Std. 10^5	Std. 10^2										H_2O
Tf	Std. 10^5	Std. 10^2										H_2O
Lac	Std. 10^5	Std. 10^2										H_2O

Figure 3: Template for the microtitre plate used for the Real-Time PCR runs

The PCR Mix used for the Real-Time PCR consisted of the following in each well:

2X Master Mix	**12.5 µl**
Forward Primer	2.5 µl
Reverse Primer	2.5 µl
Probe	2.5 µl
Aqua dest.	2.5 µl

Table 7: Real-Time PCR Mix

The above mix was prepared under sterile conditions in the 'master mix room' which is free of DNA. A total of 22.5 µl was pipetted into each of the 96 wells. The patient sample DNA was added to the above in a separate room. The microtitre plate was sealed with a round cap mat and placed in the thermocycler for the PCR run.

The PCR protocol was as follows:

Cycle	Step	Temperature	Time
1	1	50°C	10 min
2	1	95°C	10 min
3-47	1	95°C	15 sec
	2	60°C	1 min

Table 8: Real-Time PCR Run

PCR primers:

The alignment of 16S rDNA gene sequences by the software program MegAlign (DNASTAR, Madison, USA) facilitated the identification of species-specific regions useful as targets for the PCR. For the final design of specific TaqMan primers and probes the Primer Express Software (Applied Biosystems, Foster City, CA, USA) was used. All primers and probes were synthesised by Metabion (Martinsried, Germany). Only the probe for Tf was synthesised by Eurogentec S.A. (Seraing, Belgium).

4.5.4 Quantification of the PCR

Generation of the standards was essential to be able to quantitatively analyse the bacterial DNA in the samples (**Fehler! Verweisquelle konnte nicht gefunden werden.**). At the same time, the standard curves are a beneficial tool

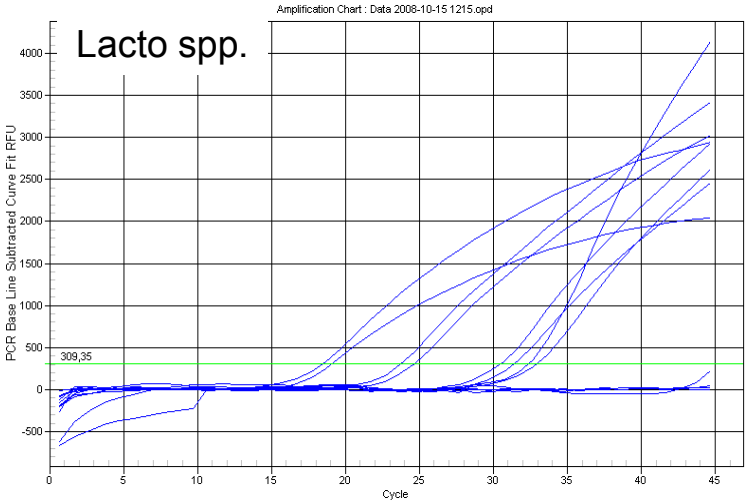
to help determine the quality and efficiency (E) of an assay (Dorak, 2007b). The standards were constructed by adding 5 µl of the successively diluted template (10^7 to 10^1) to 495 µl of 10 mM Tris/ HCl, pH 7.5 – 8.0 and running this in a conventional PCR. Sensitivity and the lower limits of the TaqMan PCR reactions were measured using the pGemT-cloned amplicons as template DNA in 10-fold dilutions ($2.5x10^5$ to $2.5x10^1$ copies/PCR). All values were measured in duplicates and linearity was reproduced in a second run.

The specificity of each TaqMan PCR set was proven in silico by Blast homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and using DNA from different oral bacteria. Blast analysis revealed that in each TaqMan PCR only DNA of the respective species which was homologous was amplified and testing of the DNA templates of oral bacteria affirmed the specificity.

The efficiency of a PCR amplification is determined from the slope of the log-linear portion the calibration curve (Bustin et al., 2009). In the calibration curve the concentration of the initial template is plotted on the x axis and C_t is plotted on the y axis. PCR efficiency is calculated as follows:

$$\text{Efficiency} = 10^{-1/\text{slope}} - 1$$

The theoretical maximum of 100% of efficiency indicates that the amount of product doubles with each cycle. At this point, the slope is -3.32. Another indicator of the quality of the assay is the r^2 or the coefficient of determination. This is a measure of the precision of the dilutions and the accuracy in pipetting during the preparation of the PCR. Ideally a perfect assay would have a slope of -3.32 (100% efficiency) and a r^2 of 1.00.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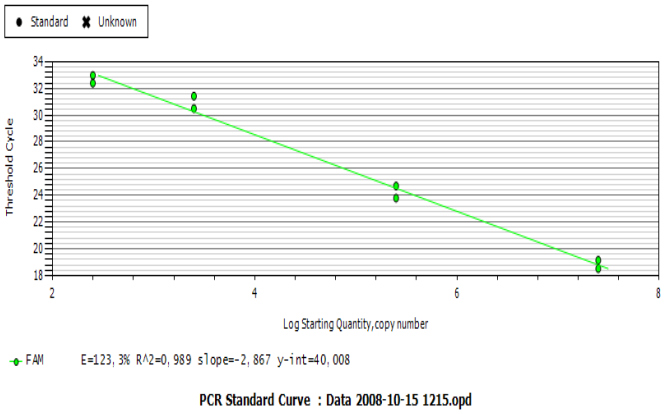
Lacto spp.
PCR Base Line Subtracted Curve Fit RFU
309,35
Cycle


Standard
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Threshold Cycle
Log Starting Quantity,copy number
FAM
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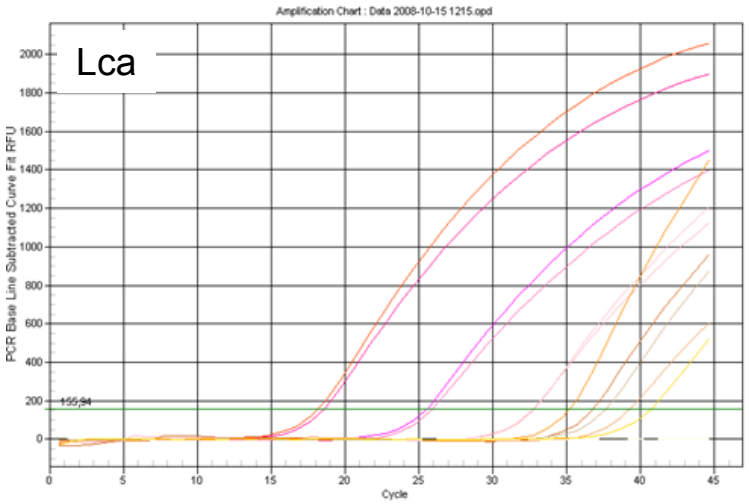
PCR Standard Curve : Data 2008-10-15 1215.opd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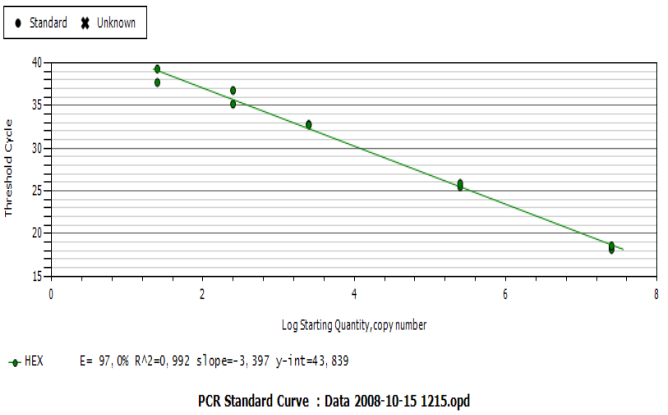
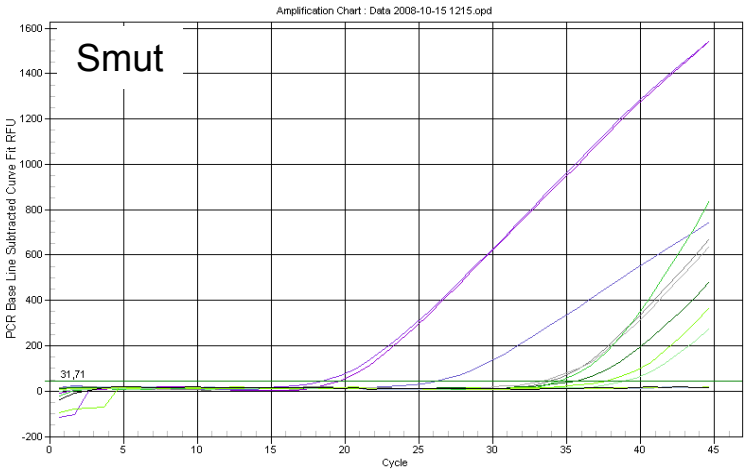
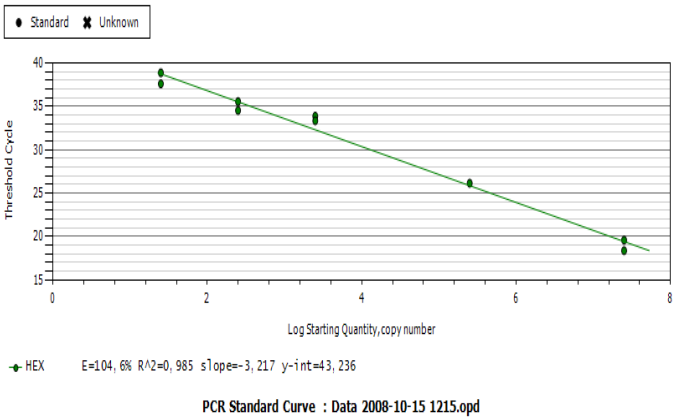
Lca
PCR Base Line Subtracted Curve Fit RFU
155,94
Cycle


Standard
Unknown
Threshold Cycle
Log Starting Quantity,copy number
HEX
E= 97, 0% R^2=0, 992 slope=-3, 397 y-int=43, 839
PCR Standard Curve : Data 2008-10-15 1215.opd


Amplification Chart : Data 2008-10-15 1215.opd
Smut
PCR Base Line Subtracted Curve Fit RFU
31,71
Cycle


Standard
Unknown
Threshold Cycle
Log Starting Quantity,copy number
HEX
E=104, 6% R^2=0, 985 slope=-3, 217 y-int=43, 236
PCR Standard Curve : Data 2008-10-15 1215.opd

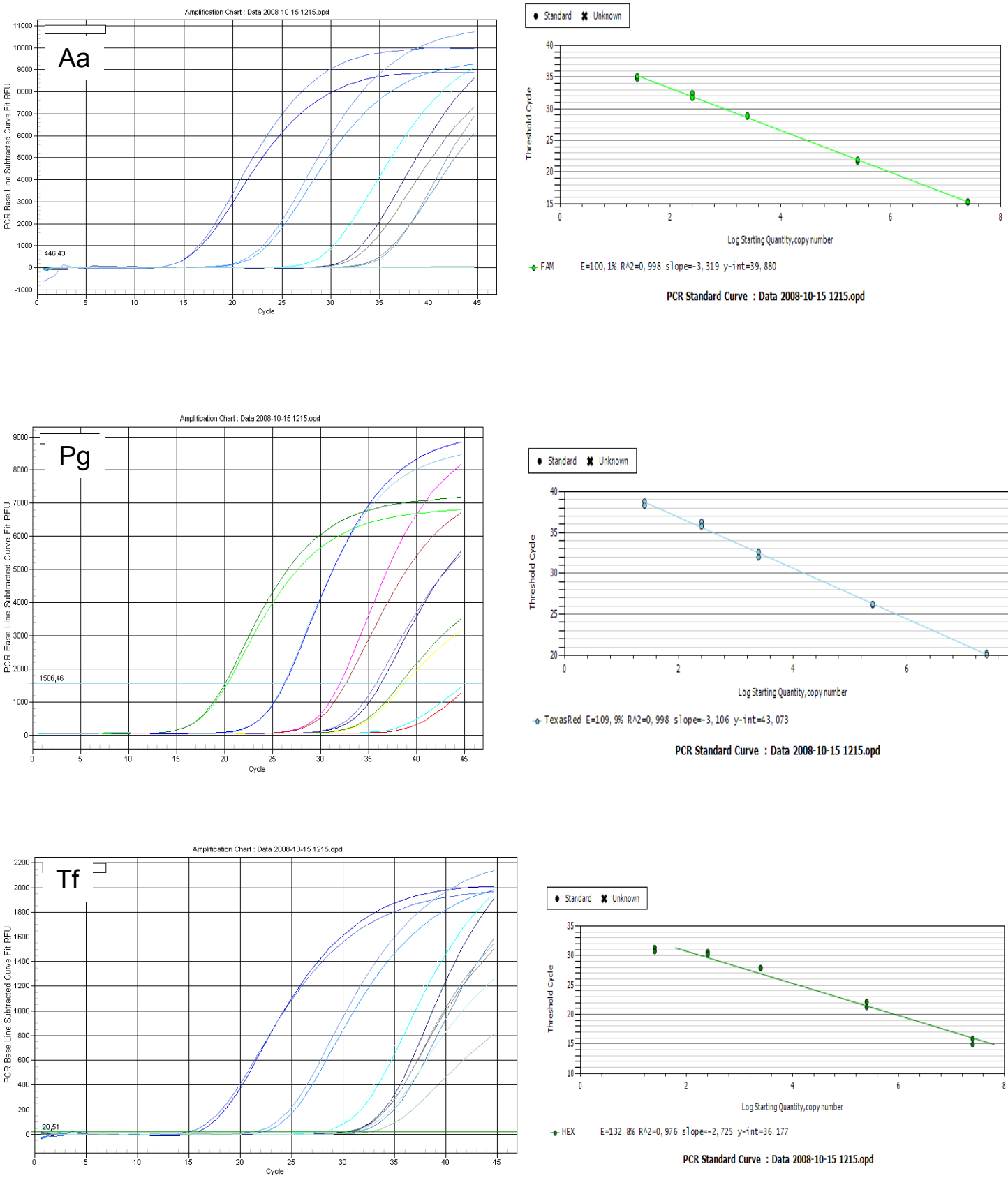


Figure 4: Generation of the standard curves for the investigated bacteria

4.6 Statistical Analysis

Raw data collected from the questionnaires and the clinical examination was organised and categorised for appropriate analysis. All the analysis of the data was done using SPSS 18 for Windows software.

Data was checked for normal distribution with the Kolmogorov-Smirnov-Test. The data was then analysed using the Kruskal- Wallis and Mann-Whitney U Test. The difference in the dental status i.e. the DMFS/dmfs Index, PBI and Silness and Löe index between the 3 groups were evaluated. The counts of the bacteria in the 3 groups were compared with Kruskal- Wallis and the Mann Whitney U Test.

It was decided to have 100 children in each group in order to have 80/ 85% chance of detecting a difference of 0.5 at the 5% level of significance.

Prior to commencement of the study, the examiner was calibrated on a series of patients to ensure validity of the clinical measurements. Kappa was performed to measure the intra-examiner reliability. A kappa value of 1 for the DMFS Index, 0.975 for Silness and Löe index and 0.85 for the PBI was obtained ensuring a high mean percentages agreement (80% - 100%).

5 Results

5.1 Results of the demographic data collected for the study

5.1.1 Age, socioeconomic status and ethnic origin

Children and adolescents from all the three groups were matched for age and sex. The average age of the participants in the group A (PKU) was 8.5 years, in group B (DM) was 11.5 years and in group C (control/ healthy) was 10.5 years.

In all three groups, A, B, and C, parents of the majority of the children and adolescents belonged to the middle-class of society, followed by a lesser percentage of the parents belonging to the upper-class and only a very small percentage belonged to the lower class (Table 9). 1, 3 and 2 persons in groups A, B and C respectively, failed to fill out the socio-economic part of the questionnaire completely and therefore could not be included in the analysis. In both groups B and C more than 70% of the children and adolescents were of German origin with the rest of the sample population being of foreign origin. In group C 90% of the participants were of German origin and the rest of foreign origin (Table 10).

Group (N)	Lower-class (%)	Middle-class (%)	Upper-class (%)
A (37)	13.2	57.9	26.3
B (97)	9	54	34
C (98)	4	49	45

Table 9: Percentage of the socioeconomic status of the parents of the children and adolescents in all three groups A, B, and C

Group (N)	German origin (%)	Foreign origin (%)
A (38)	71.1	28.9
B (100)	77	23
C (100)	90	10

Table 10: Percentage of the ethnic origin of the children and adolescents in all the three groups A, B and C

5.2 Dental status of the children and adolescents

5.2.1 dmfs Index

A comparison of the dmfs index for the primary dentition revealed that the children in group A had an astonishingly high value as compared to the other two groups. A statistically significant difference was found between the three groups. Primary teeth of the children in group A showed a mean (± Standard deviation) dmfs index value of 4.18 (±7.46) whereas those of group B and C showed values of 1.38 (± 5.33) and 3.86 (±7.68), respectively. Thus the children in group B demonstrated the lowest mean dmfs index values (Table 11).

Group	N	Minimum	Maximum	Mean	Standard Deviation
A	37	0	32	4.18*	7.46
B	100	0	50	1.38*	5.33
C	100	0	56	3.86*	7.68

Table 11: dmfs index values for groups A, B and C. * indicates statistically significant difference between the groups, $p \leq 0.05$.

5.2.2 DMFS Index

A comparison of the DMFS index values for the secondary dentition revealed no statistically significant difference between the children and adolescents in the three groups. The participants in group A were found to have the highest mean (± Standard deviation) DMFS index value of 3.16 (±8.92), closely followed by the participants in group B with 3.08 (±11.38), and by those in group C with a mean value of 2.57 (±7.11) (Table 12).

Group	N	Minimum	Maximum	Mean	Standard Deviation
A	37	0	51	3.16	8.92
B	100	0	88	3.08	11.38
C	100	0	61	2.57	7.11

Table 12: DMFS index values for groups A, B and C

5.3 Oral hygiene and gingival health

The oral health of the children was evaluated by determining the Silness and Löe Index and the Papillary bleeding Index.

5.3.1 Silness and Löe Index

A comparison of the three groups for the Silness and Löe Index revealed a small statistically significant difference between the groups, with participants of group B showing higher values than those in group A and C (p=0.04) (Table 13). A comparison between groups B and C showed a more significant statistical difference between the two groups (p=0.01).

Group	N	Minimum	Maximum	Mean	Standard Deviation
A	38	0	3.55	0.94*	0.86
B	100	0	3.00	1.05**	0.75
C	100	0	3.00	0.79*,**	0.70

Table 13: Silness and Löe index values for groups A, B and C. □ indicates small statistically significant difference between the 2 groups, ** indicates statistically significant difference between the two groups, $p \leq 0.05$.

5.3.2 Papillary bleeding Index

No statistically significant difference was found between the three groups for the papillary bleeding index (Table 14). However, comparison of participants in group B with those in group C showed a very small statistically significant difference between the two groups, with the PBI score values being slightly elevated in group C (p=0.04).

Group	N	Minimum	Maximum	Mean	Standard Deviation
A	37	0	1.71	0.24	0.46
B	100	0	2.57	0.30*	0.48
C	100	0	1.93	0.17*	0.32

Table 14: Papillary bleeding index values for groups A, B and C. * indicates statistically significant difference between the two groups, $p \leq 0.05$.

5.4 Microbiological results in the children and adolescents

The oral microorganisms investigated in the study were Streptococcus mutans (Sm), Lactobacillus species (Lac), Lactobacillus casei (Lca), Aggregatibacter actinomycetemcomitans (Aa), Porphyromonas gingivalis (Pg) and Tannerella forsythensis (Tg). A comparison of all the bacteria between the three groups revealed a statistically significant difference between Lac, Lca and Pg (Table 15). Counts of Lac were found to be the lowest in the group A. Group B showed the highest counts of Lca but lowest for Pg. Group C showed the lowest counts for Lca and the highest counts for Lac and Pg.

Group	Lac	Lca	Sm	Aa	Pg	Tf
A	21.18* (40.46)	1.38x 10^{2}* ($8.27x10^{2}$)	$3.18x10^{10}$ ($1.96x10^{11}$)	1.26 (3.27)	$2.66x10^{3}$* ($1.64x10^{4}$)	$5.41x10^{3}$ ($2.14 x 10^{4}$)
B	$1.04x10^{2}$* ($3.70x10^{2}$)	$1.29x10^{3}$* ($8.50x10^{3}$)	$1.27 x 10^{7}$ ($1.26 x 10^{8}$)	$1.22x10^{8}$ ($1.22x10^{9}$)	54.64* ($1.30x10^{2}$)	$1.53 x 10^{5}$ ($8.51x 10^{5}$)
C	$3.11x10^{3}$* ($3.00x10^{4}$)	31.43* ($1.29x10^{2}$)	$6.20 x 10^{3}$ ($4.34 x 10^{5}$)	$3.67x10^{7}$ ($2.68x10^{8}$)	$6.76x10^{4}$* ($6.55x10^{5}$)	$7.55 x 10^{5}$ ($7.27x 10^{6}$)

Table 15: Mean (± Standard deviation) values of the copies/ mg of the sample for the microorganisms in groups A, B and C. * indicates statistically significant difference between the groups, $p \leq 0.05$.

Comparison of the levels of the three bacteria between group A and B showed that Pg was significantly lower and Lca was higher in the group B children (type 1 diabetes). Comparison of the levels of the three bacteria between group A and C showed that levels of Lac and Pg were significantly lower in group A

children (PKU). Comparison of groups B and C showed that group B had lower Lac and Pg levels but higher levels of Lca.

6 Discussion

6.1 Study design

This study was carried out with the aim of evaluating the oral status and the microorganisms in patients with type 1 diabetes and PKU. It was proposed that the two groups of patients have diets converse to each other and thus present with a contrary risk for caries and periodontal disease. On the other hand, according to newer disease management protocols for type 1 diabetes, dietary restrictions for the patients have been relaxed. This is enabling them to lead a more normal life in terms of the diet. Therefore, our secondary objective was to determine whether the original proposition still holds true. This in mind, three groups of patients were examined, viz. children with PKU, type 1 diabetes and children with no systemic disease. The sample of PKU children was smaller, as the disease is relatively rare. All other studies, assessing the oral health of children with PKU had similar or smaller sample sizes. (Lucas et al., 2001; Kilpatrick et al., 1999; Winter et al., 1974; Johnson et al., 1970)

Data regarding the socioeconomic status, and ethnic origin of parents and children was collected with the help of a questionnaire designed by the Robert Koch Institute (Berlin, Germany) and used for the German Health Interview and Examination Survey for Children and Adolescents (KiGGS), 2003-2006. The social status scaling index employed in the questionnaire is well established and has been applied in German national health surveys (Winkler and Stolzenberg, 1999). Therefore it was a valid and recognized questionnaire and index, with a sure degree of reliability that was applied in our study.

The DMFT index is the most commonly employed index to assess the dental status among a population. It is applied in studies worldwide and recommended by the WHO for dental surveys (WHO, 1997). The gingival health and oral hygiene were evaluated with the help of Papillary Bleeding Index and the Silness and Löe plaque index. The PBI has the advantage of providing a sufficiently precise picture of the gingival health with relatively little investment

of time. Although it is quick and accurate, it is slightly restricted as it only assesses the health of the gingival papilla. The Silness and Löe plaque index records the amount of plaque at the gingival margin, thus proving to be more useful an index to determine the risk of development of caries and periodontal disease than another index that records the extension of plaque over the complete tooth surface. Concurrently, as it does not necessitate the use of a disclosing agent it is a rapid and tidy means of assessing the oral hygiene habits of a patient (Rebelo & Corre□a de Queiroz, 2011).

6.2 Microbiological testing

Several methods are used for the detection of oral microorganisms, viz. traditional bacterial culture methods, immunological assays, enzymatic method and since the late 90's molecular biological methods. Real-time PCR is fast replacing traditional culture based methods as a qualitative and quantitative diagnostic technique in microbiology. Direct counting of individual cells using the microscope is probably the oldest method used for microbial quantification. The above techniques were considered as the 'gold standard' for the identification and quantification of bacteria. However, a significant drawback in these methods is the time required, often taking up to several days for a result. Furthermore, these traditional methods also are considered to sometimes have low sensitivity, be able to lowly or poorly grow viable organisms, have narrow detection ranges, be complex to interpret and have high levels of background and non-specific cross-reactions. These methods, on the other hand, supply important data for new, uncharacterized or atypical organisms for further research (Dorak, 2007a). The improvement in molecular biological techniques over the past decades has led to revolutionary changes in diagnostic methods in microbiology, initially with conventional PCR and now with the highly accurate and specific real- time PCR. The sensitivity of real-time PCR enables the detection of less than 10 cells with sensitive background regulations (Dorak, 2007a). Detection of the oral microorganisms in this study was conducted by real-time TaqMan PCR. Due to their rapidity, sensitivity and specificity, PCR

based diagnostic methods have gained in popularity for the diagnosis and detection of oral bacteria. Very minute quantities of DNA, which may even be old or degraded, are sufficient to amplify and quantify the required DNA accurately. However, the sensitivity of PCR is also one of its major drawbacks as very minor amounts of foreign DNA can contaminate the sample and distort the results (Suzuki et al., 2005). PCR methods are, however relatively expensive as compared to traditional culturing methods. Nonetheless, when considering the cost-benefit ratio, PCR methods definitely have the upper hand, in terms of time and accuracy. Real-time PCR with highly specific primers and TaqMan probes enables the continuous monitoring of DNA amplification, eliminating the need for time consuming gel electrophoresis (Crockett & Wittwer, 2001).

Another advantage of the TaqMan PCR is that multiple TaqMan probes and primer sets can be used in different assays to differentiate between closely related sequences. Moreover, probes can be labelled with different fluorophores, thus developing a multiplex real-time PCR protocol allowing different targets to be co-amplified and quantified within a single reaction.

16S rRNA sequences are usually used as primers in PCR based diagnostic methods for the detection of oral microorganisms (Ashimoto et al., 1996; Yoshida et al., 2003). Nevertheless, these primers may prove inadequate for quantitative analysis as every microorganism may contain a certain amount of these sequences and dental plaque has several closely related species. Hence, primers based on a single copy gene sequence, which are more specific for a target bacterium, are considered a better option for this purpose (Morillo et al., 2004). However, isolation of specific nucleotide sequences from the genes of organisms for which species-specific proteins have not already been characterized proves to be challenging (Suzuki et al., 2004). Out of the five oral microorganisms investigated in this study, only the species-specific TaqMan probes and primers for Lactobacillus casei were self-designed. As the species specific proteins for remaining bacteria had been previously characterized in other studies it was possible to take the sequences for these from the literature (Morillo et al., 2004; Byun et al., 2004; Price et al., 2007).

6.3 Results

6.3.1 Type 1 diabetes and dental caries

In the present study, it was found that the mean dmfs index value in the diabetic children was statistically significantly lower than in the healthy children and the children suffering from PKU. The mean DMFS index values, however, were similar in the diabetic, PKU and healthy children. Comparable findings were shown in a recent cross-sectional study, in a sample with a similar age range as the present study (Tagelsir et al., 2011). Correspondingly, they found a notable difference in the mean dmfs value between healthy children and type 1 diabetic children, with the diabetic children showing lower values. Little or no difference was found in the mean DMFS value. Interestingly, at the same time the study also revealed a high level of untreated decay in diabetic children. The study clearly outlined a high dental treatment need in diabetic children.

As mentioned earlier in the literature review, results for dental caries experience have been particularly inconsistent. On one hand, older and newer studies have been able to show a lower prevalence of caries in type 1 diabetic children than in healthy controls (Orbak et al., 2008; Goteiner et al., 1986) and on the other, a number of other studies (Twetman et al.,1989; Swanljung et al., 1992; Siudikiene et al., 2008; Siudikiene et al., 2006; Lalla et al. 2006a; Edblad et al., 2001) have found results analog to the present study where the diabetics have a similar caries rate as the healthy controls. Most of these studies have, however, been conducted on samples with an older age group. Thus the results provided by the studies are mainly for adolescents. Many of them also discuss this age group as one of emotional turmoil, with problems in compliance, not only with oral hygiene, but also with diabetes therapy. This can lead to problems with maintenance of metabolic control of the disease, which causes physiological complications (Syrjälä et al., 2002). The same reasoning, coupled with a diabetic diet which nowadays is almost similar to that of a healthy child, may be found explicable to support the result in the present study of similar

caries rate in the permanent teeth of the diabetics and that of the healthy controls or the children with PKU.

Some studies have been able to highlight a positive correlation between metabolic control of the diabetes and dental caries, whereby poorly controlled patients show higher levels of caries (Miko et al., 2010; Karjalainen et al., 1997; Saes Busato et al., 2010; Bolgül et al., 2004; Canepari et al., 1994). Miko et al. stated that the increase in caries levels in poorly controlled adolescents, notwithstanding their sucrose-free diet, could be attributed to their faulty tooth brushing habits or to their disregard for the proper metabolic control of their disease. Poor control resulted in a decrease in the local oral pH and in the cleansing effect of saliva. This, along with poor oral hygiene, resulted in the development of dental caries (Miko et al., 2010). In a 3 year follow-up study, Twetman et al. found that diabetic children and adolescents with poor metabolic control developed 3 times more carious lesions compared to those with good metabolic control (Twetman et al., 1992). They justified their findings by explaining that elevated glucose concentrations, both in stimulated and resting saliva, were found in poorly controlled metabolic diabetics. This released excess glucose into the oral cavity via saliva and the gingival crevicular fluid, which lead to the formation of a 'sugar-rich' biofilm. This biofilm augments plaque growth and establishes ecological conditions favourable for aciduric bacteria, thus supporting the development of dental caries. The maintenance of metabolic control is reliant on the patient's capability to perform self-care procedures such as, regular insulin injections and adhering to the necessary dietary restrictions. However, at times, it is possible that the maintenance of good metabolic control might be affected by genetic factors (Karjalainen, 2000). Similarly, self-care procedures are vital in the prevention of dental caries and periodontal diseases. An interesting aspect was depicted by a group of Finnish authors, who found that promotion of oral health in diabetics positively influences diabetes adherence (Kneckt et al., 1999; Syrjälä et al., 2002). Consequently, maintenance of oral health influences maintenance of diabetic control, which in turn has an effect on the development of caries.

Contrary to the above, there are studies which have shown higher caries levels in children and adolescents with diabetes irrespective of the metabolic control (López et al., 2003). It should, however, be noted that the sample size in the study conducted by López et al. was rather small. Hence, it would be prudent to exercise caution while transferring these results to a larger population. The majority of the patients (> 88%) included in the present study had a good to moderate control of diabetes, with all patients having a HbA1 value of over 9% being considered as poorly controlled. Therefore, it is unlikely that the results of the present study have been influenced by the metabolic control of the disease.

6.3.2 Type 1 diabetes, oral hygiene and gingival health

In the present study, the oral or gingival health of the children and adolescents with diabetes was found to be slightly worse than that of the healthy children and that of the children with PKU. This finding is congruent with the results of several other studies examining the gingival or periodontal health of children with diabetes (Goteiner et al., 1986; Sandholm et al., 1989b; Pinson et al., 1995; Novaes et al., 1991; Lopez et al., 2003; Lalla et al., 2006a; Saes Busato et al., 2010; Tagelsir et al., 2011). Furthermore, some studies have been able to show a significant worsening of the gingival health in patients with poor control of diabetes (Karjalainen et al., 1997; Tervonen et al., 1986; Aren et al., 2003). Karjalainen et al. examined newly diagnosed children in the hospital and following discharge from the hospital. The authors were able to find an improvement in the gingival bleeding after commencement of treatment, therefore establishing a causal relationship between metabolic control of diabetes and gingival health. In addition, they were able to show that poorly controlled diabetic children and adolescents with a longer disease duration had significantly more gingival bleeding than the moderately and well controlled ones. Strictly seen, however, it must be stated that the value set for HbA1 for well-controlled diabetics in the study was very high (HbA1 < 10%) and does not correspond with the actual guidelines. In later and current studies this value would also include patients with poor metabolic control of the disease.

Nevertheless, there are studies that have not been able to show any differences in the plaque and gingival index or the gingival bleeding between diabetic children and adolescents and non-diabetics (Firatli et al., 1996, Firatli, 1997; Barnett et al., 1984; Siudikiene et al., 2006, 2008; Sbordone et al., 1998). Siudikiene et al. found that the oral hygiene of both the healthy and diabetic children worsened over a 2 year period. In a 5 year follow-up study, Firatli found no differences between the plaque and gingival indices of the diabetic and non - diabetic children and adolescents. Furthermore, they were unable to reveal a positive correlation between the plaque index, gingival index and duration of diabetes but were able to observe this between the clinical attachment level and longer duration of diabetes. The authors stated that as diabetes is known to modify the clinical status of periodontal tissues by its altered immunological response, increased inflammatory response, altered collagen turnover and predisposition to infections, the longer duration of diabetes could well increase clinical attachment loss through periodontal complications. Barnett et al. substantiated their findings of no differences between the diabetics and non - diabetics with the explanation that there are differences in susceptibility to periodontal disease among diabetic populations, with subpopulations of varying sizes, that have an increased disease susceptibility. Furthermore, they suggested that racial and sexual differences may also account for differences in susceptibility. In the present study, however, this cannot be considered as one of the influencing factors as the majority of the population was of the same ethnic origin (German) and the division of gender was almost equal.

Studies regarding the gingival or periodontal health of diabetic patients are abundant and therefore, it is an easy task to find studies with results that match that of the present study and at the same time a number of studies that show dissimilar results. In 2006, Khader et al. published a meta-analysis examining the periodontal status of diabetics compared with non-diabetics. The meta-analysis included all studies conducted with type 1 and 2 diabetics between the years 1970 and 2003. The authors came to the conclusion that although diabetics have a significantly worse oral hygiene, higher severity of gingival disease and periodontal disease they present with a similar extent of gingival

and periodontal disease as the non-diabetics. This result was supported by the observation that regular dental care and tooth brushing habits in diabetics may be similar to that of non-diabetics (Khader et al. 2006).

Sepällä et al. took biopsies from gingival connective tissue of type 1 diabetics and studied them for cellular and vascular changes that might be able to explain the altered reaction of the gingival tissue in diabetics. They were unable to detect vascular changes or specific characteristics of inflamed connective tissue in well-controlled long-term diabetics under similar plaque conditions. Patients with poor control of diabetes, however, often showed swollen and proliferated endothelial cells. They found that patients with long-term poor control of diabetes showed connective tissue changes, cellular and vascular, indicative of increased catabolism rather than anabolism in the gingiva. The authors provided an indefinite conclusion, stating that “one can speculate whether the inflammatory response to a minor amount of plaque in long-term poorly controlled diabetic subjects results in a plasma cell-dominated infiltrate with loss of collagen and enlargement of vascular membranes” (Seppälä et al.,1997). In an experimental gingivitis model, Salvi et al. found that both diabetics and non-diabetics reacted equally with gingival inflammation to experimental plaque accumulation. However, the type 1 diabetics, develop an quicker and greater inflammatory response to a similar bacterial challenge (Salvi et al., 2005). A few years later Salvi et al. reported that the gingival cervicular fluid biomarker levels for IL-1b and MMP-8 were notably elevated in type 1 diabetics compared to their healthy counterparts. This, however, did not arise due to differences in the plaque index or the microbial composition of the two groups (Salvi et al., 2010).

As mentioned above (Firatli et al., 1997), several theories have been postulated for altered periodontal tissue in diabetics. A consensus as to a definitive cause for the increased severity of periodontal disease in these patients has, however, till date not been reached. Research continues in the field with aims to cover all aspects of both multifactorial diseases. One study suggests that periodontal destruction can start very early in life in diabetics, becoming more pronounced as children progress into adolescence (Lalla et al., 2006a). Despite the fact that all cases of gingivitis do not lead to periodontitis, periodontitis is preceded by

gingivitis. Therefore, children and adolescent with type 1 diabetes should be regularly examined for signs of gingivitis.

6.3.3 Type 1 diabetes and oral microorganisms

In the present study, children and adolescents with type 1 diabetes showed the highest counts of Lactobacillus casei but lowest for Prophyromonas gingivalis. The children with no systemic disease showed the highest counts of Lactobacillus species and Prophyromonas gingivalis. The results of the present study are in part comparable to results of other studies as no other study has investigated the specific bacteria relevant to caries and periodontal disease together, using PCR, in a group of type 1 diabetic children and adolescents, as in this study. The advantages of molecular biological methods in investigating the oral microflora have already been mentioned above. Some studies have investigated solely Streptococcus mutans and Lactobacillus species (Siudikiene et al., 2008; Siudikiene et al., 2006; Twetman et al., 1989; Twetman et al., 2002; Karjalainen et al. 1997; Bolgül et al. 2004). Either traditional laboratory culture methods or a commercial kit were used in these studies to determine the colony forming units/ ml of bacteria. Moreover, all the studies used saliva as a sample to determine the bacterial count. Saliva does provide a broad picture of the oral bacterial load but is a weak tool to investigate bacteria that are potentially periodontal pathogenic. Therefore, in the present study the sample for determination of the microorganisms was taken from the cervical region (supragingival plaque) and gingival sulcus. Karjalainen et al. and Siudikeine et al. did not observe any differences in counts for Streptococcus mutans and Lactobacillus species between the diabetic and non -diabetic children and adolescents. In one of the studies they did, however, show an increase in the counts for the above bacteria in patients with poor diabetic control (Siudikiene et al., 2006). The same result could also be reported by Bolgül et al. (2004). In 1989, Twetman et al. reported low levels of Lactobacilli in diabetic patients. The number of Lactobacilli were found to be positively correlated to concentration of glucose in saliva. The authors suggested that it is essentially the dietary

treatment of diabetic children and adolescents which gives rise to a reduction of lactobacilli in saliva but does not affect the Streptococcus mutans count. Twetman et al., similar to the present study, reported high levels of lactobacilli in the saliva of diabetic patients (Twetman et al., 2002). According to the study group, this observation can be attributed to the less restricted diet of today's diabetics. Likewise, our finding of no statistically significant difference in the Streptococcus mutans and Lactobacillus species counts and increased counts of Lactobacillus casei in the type 1 diabetics may be imputed to the relatively similar dietary lifestyles of the diabetics and healthy children.

Very few studies can be found that investigate the periodontal pathogenic bacteria in children and adolescents with type 1 diabetics (Sbordone et al., 1995; Sbordone et al., 1998; Sandholm et al., 1989a; Mashimo et al., 1983). Sbordone et al. were able to identify a cluster in their sample with high levels of Porphyromonas gingivalis and Capnocytophaga species. P. gingivalis was present in the patients at levels much higher than the reported critical level (0.1%) that defines a periodontal site with non-progressing disease. Nevertheless, none of the clusters exhibited any further periodontal breakdown over the 3-year interval of the study. The authors suggested that the presence of these microorganisms does not pose a risk for periodontal breakdown in diabetic patients (Sbordone et al., 1998). Sandholm et al. found higher proportions of gram-negative rods and total gram-negative bacteria in type 1 diabetic patients who presented with a higher degree of gingivitis, even though they had similar plaque index scores to the non-diabetics (Sandholm et al., 1989a). The results of the present study cannot be confirmed by any of the above mentioned studies as the diabetic patients in this study were found to have the lowest counts of Pg and no statistically significant difference was found in the counts of Aa and Tf. Consequently, no microbiological reasoning for the slightly raised level of gingivitis in the diabetics can be provided. However, the study does provided proof that the oral microflora of the diabetics does not differ much from that of healthy subjects.

6.3.4 PKU and dental caries

The results of the present research showed that the mean dmfs index value in the group of children suffering from PKU was statistically significantly higher than in the healthy children and the diabetic children. The mean DMFS index values, however, were similar in all three groups. As mentioned earlier in the literature review, studies investigating the dental status and oral health of children with PKU are few and far between. The last study published on the subject was over a decade ago in 2001 (Lucas et al., 2001). Similar to the present study, this study examined forty-one children suffering from PKU for dental caries, plaque and gingivitis indices and caries related oral flora. The investigation was unable to reveal any significant difference in the mean dmfs or DMFS values between the PKU children and the controls. An Australian group of workers evaluated the oral health of PKU children and concurrently investigated the erosive potential of 5 amino acid supplements regularly taken by the children. Interestingly, the study group found that the titratable acidity of the flavoured amino acid supplements was significantly higher than both their unflavored counterparts and Coca Cola (Kilpatrick et al., 1999). They were able to report a rather low caries rate but higher levels of tooth wear. In fact, the study found that the whole sample showed a lower caries rate than that reported overall for the Australian children. In both the above mentioned studies, the authors attributed the reported caries related findings to the use of fluoride supplements and water fluoridation. In an attempt to elucidate the low caries prevalence in the presence of a highly cariogenic diet, Kilpatrick further surmised the role of phenylalanine in limiting the growth of oral plaque microorganisms, as suggested by Winter et al. (1974). Older studies have likewise all revealed similar or lower caries experience in PKU children despite their known use of highly acidic supplements and a diet rich in carbohydrates and fat (King, 1969; Johnson et al., 1970; Winter et al., 1974). In the present study, the children with PKU had an equal intake of fluoride supplements as the healthy control children, which was considered to be a moderate to good intake of fluorides (data not shown). Nevertheless, this does not provide an explanation for the higher caries experience in the primary dentition of the

children with PKU compared to the healthy or the diabetic children. It may be speculated that in the earlier years of the child's life, priorities of the parents of children with PKU lie more in maintaining an optimal general health and daily functioning of the child rather than focusing on prevention of oral disease.

6.3.5 PKU, oral hygiene and gingival health

In this study, the oral hygiene and gingival health, as estimated by the plaque index by Silness and Löe and the papillary bleeding index, revealed no differences between the children with PKU and the healthy children and diabetics. Contrary to the results of the present study, Lucas et al. found greater mean plaque score in children with PKU and no difference in the gingival inflammation or spontaneous bleeding between PKU children and the controls (Lucas et al., 2001). On the other hand, Kilpatrick et al. found exactly the opposite viz., there was no difference in the plaque and oral debris between PKU children and the controls but the PKU children exhibited significantly more gingival inflammation than the controls (Kilpatrick et al., 1999). The authors, however, did not justify this finding.

6.3.6 PKU and oral microorganisms

The results of the present study demonstrated significantly lower levels of Lactobacillus species in children with PKU than in the healthy and diabetic children but no difference in the any of the other investigated bacteria. These results differ only slightly from those of Lucas et al., who examined the saliva and plaque of their sample group for Streptococcus mutans and Lactobacilli by traditional culture methods. They reported no differences between the bacterial counts of the children with PKU and control subjects (Lucas et al., 2001). No other study has documented the oral microflora in children suffering from PKU. The results of this study can substantiate most of the findings on a microbiological basis with no difference in the dental status, oral hygiene and

gingival health between the children with PKU and the diabetic and healthy older children and adolescents.

7 Conclusions

The primary dentition in the PKU children revealed an astonishingly higher prevalence of dental caries than that of the diabetic or healthy children. The permanent dentition was, however, found to have the same prevalence of caries as in the diabetic and healthy children and adolescents. At the same time, oral hygiene and gingival health was found to be comparable to that of the healthy children and adolescents.

The primary dentition of children with type 1 diabetes was found to have a lesser prevalence of caries than the healthy or PKU children. The same positive finding could not be confirmed regarding the permanent teeth of the diabetic population in this study. The permanent teeth were found to have the same prevalence of caries as the healthy and the PKU children. Noteworthy, was the result that although the oral hygiene and gingival health was slightly worse in the diabetic children and adolescents they did not harbor more periodontal pathogenic bacteria than the healthy or PKU children, in fact the diabetic group revealed the lowest levels of Prophyromonas gingivalis. The diabetic children did, however, show higher levels of Lactobacilli casei than the children with PKU and healthy children, which may suggest a relationship between a shift in the oral microflora and the relatively unrestricted dietary habits of the diabetic patients. The children suffering from PKU, on the other hand, revealed significantly lower levels of Lactobacillus species compared to the healthy and diabetic children and adolescents.

Thus the null hypothesis of our study can, only in part, be rejected and in part, the alternative hypothesis that children (not adolescents) with PKU possess a higher caries rate than their healthy or diabetic counterparts and that diabetic children (not adolescents) have a lower caries rate and but a slightly higher risk of developing periodontal disease, can be accepted.

The oral disease patterns observed in this study might possibly be ascribed to the focus of parental attitudes towards the maintenance of normal general health in view of the systemic disease, thus disregarding oral health in earlier

stages of the child's life. It is known that prevention of dental caries and periodontal diseases is chiefly dependent on the patients' home care measures and regular dental visits. Accordingly, in view of the higher risk for caries (PKU) and periodontal disease (diabetes) at an earlier age for these children, it is proposed that both groups of child patients, i.e. diabetics and PKU, be encouraged to seek early dental advice and be provided with targeted dental education.

The somewhat small sample size of the PKU children, the broad range in the age of the participants and the cross-sectional design of this study may be seen as the limitations of the study. These restricting factors prevent us from drawing definitive conclusions regarding the impact of the systemic diseases, PKU and type 1 diabetes, on the overall oral health of the child and adolescent patients. Nonetheless, in light of the present findings, additional vital information is likely to be attained by a planned longitudinal study, to help disclose assuredly the relationship between the two metabolic illnesses and oral diseases.

8 References

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9 Appendix

9.1 Chemicals

Agarose	**Biozym Scientific GmbH, Hess. Oldendorf, Germany**
Bromophenol blue	Merck, Darmstadt, Germany
Dinatriumhydrogenphosphate	Merck, Darmstadt, Germany
EDTA	Sigma-Aldrich Chemie GmbH, München, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Sigma-Aldrich Chemie GmbH, München, Germany
Glucose	Merck, Darmstadt, Germany
Glycerine	Merck, Darmstadt, Germany
Yeast extract	Gibco BRL, Invitrogen GmbH, Karlsruhe, Germany
Isopropanol	Merck, Darmstadt, Germany
Kaliumacetate	Merck, Darmstadt, Germany
Magnesiumchloride	Merck, Darmstadt, Germany
Natriumchloride	Merck, Darmstadt, Germany
Tris (hydroxymethylaminomethane)	Merck, Darmstadt, Germany
TSB (Tryptic Soy Broth)	Difco Laboratories GmbH, Augsburg,Germany
PBS (Phosphate buffered saline)	Gibco BRL, Invitrogen GmbH, Karlsruhe, Germany

9.2 Questionaire

Questionnaire used in the study to collect sociodemographic data and information on oral hygiene habits and fluoride intake of the participants.

Sehr geehrte Eltern,

anbei erhalten Sie auf freiwilliger Basis einen Fragebogen, welcher für die

Analyse und Auswertung der Studie benötigt wird. Die Fragen zur Soziodemografie orientieren sich am Fragebogen des Robert Koch Institutes, der im Rahmen des Kinder- und Jugendgesundheitsurveys KiGGS eingesetzt wird.

Soziodemografische Fragen

Ihre Adresse:

1. Welchen Schulabschluss haben Sie? **Vater Mutter**

(1) Hauptschulabschluss/ Volksschulabschluss

(2) Realschulabschluss (Mittlere Reife, Fachoberschulreife)

(3) Abschluss Politechnische Oberschule (POS)

(4) Fachhochschulreife, Abschluss Fachoberschule

(5) Abitur (Gymnasium, fachgebundene Hochschulreife)

(6) Anderer Schulabschluss

(7) Schule beendet ohne Schulabschluss/ kein Schulbesuch

(8) Noch keinen Schulabschluss

2. Welchen Berufsausbildungsabschluss haben Sie?

(1) Lehre (beruflich-betriebliche Ausbildung)

(2) Berufsschule, Handelsschule (beruflich-schulische Ausbildung)

(3) Fachschule (z.B. Meister- Technikerschule, Berufs- oder Fachakademie

(4) Fachhochschule, Ingenieurschule

(5) Universität, Hochschule

(6) Anderer Ausbildungsabschluss

(7) Kein beruflicher Abschluss und auch nicht in Ausbildung

(8) Noch in beruflicher Ausbildung (Auszubildender, Student)

3. Sind Sie zurzeit berufstätig?

Zurzeit

(1)...nicht berufstätig (Hausfrau/-mann, Student, Rentner usw.)

(2)...arbeitslos - nicht beschäftigt

(3)...arbeitslos – Vollzeit beschäftigt (z.B. ‚Ein-Euro-Job' bei ALGII)

(4)...arbeitslos – Teilzeit oder stundenweise beschäftigt

(5)...vorübergehende Freistellung (z.B. Elternzeit)

(6)...Teilzeit oder stundenweise berufstätig

(7)...voll berufstätig

(8)...Auszubildender (z.B. Lehrling)

4. Wer kümmert sich um Ihr Kind, wenn Sie bei der Arbeit sind?

(1) Kindergarten/ Schule

(2) Großeltern

(3) Kindergarten, danach bei Großeltern

(4) Tagesmutter

(5) Au-Pair Mädchen

(6) Kinderhort

(7) Ehemann/-frau oder Partner

5. Bei wem lebt ihr Kind hauptsächlich?

(1) Leibliche Eltern

(2) Mutter und ihr Partner

(3) Vater und seine Partnerin

(4) Mutter

(5) Vater

(6) Großeltern oder andere Verwandte

(7) Pflegeeltern/ Adoptiveltern

(8) In einem Heim

6. Mit wie vielen Geschwistern lebt Ihr Kind zusammen?

(0) mehr als vier

(1) vier

(2) drei

(3) zwei

(4) ein

(5) kein

Fragen zur Mundgesundheit

1. Wie oft reinigt Ihr Kind sich die Zähne?

(0) nicht jeden Tag

(1) einmal am Tag

(2) zweimal am Tag

(3) mehr als zweimal täglich

2. Wann haben Sie angefangen die Zähne Ihres Kindes zu reinigen?

(0) nach drei Jahren

(1) mit zwei bis drei Jahren

(2) mit ein bis zwei Jahren

(3) mit dem ersten Zahndurchbruch

3. Wer reinigt die Zähne Ihres Kindes?

(0) das Kind selbst

(1) ich

(2) das Kind, ich reinige nach

4. Haben Sie gemerkt, dass Ihr Kind Zahnfleischblutung hat?

(0) ja, jeden Tag

(1) ja, manchmal

(2) nein

5. Wurde Ihr Kind schon einmal zahnärztlich untersucht?

(0) nein, noch nie

(1) ja, mindestens einmal

(2) ja, regelmäßig

6. Wann war Ihr Kind zum ersten Mal beim Zahnarzt?

(0) noch nie

(1) erst nach drei Jahren

(2) erst mit zwei bis drei Jahren

(3) erst mit ein bis zwei Jahren

(4) erst mit dem ersten Zahndurchbruch

7. Hat Ihr Kind schon einmal Zahnschmerzen gehabt (kein Zahnungsbeschwerde)?

(0) ja, öfter

(1) ja, einmal

(2) nein

8. Hat Ihr Kind kranke Zähne („Loch im Zahn“)?

(0) ja

(1) nein (Frage 9 überspringen)

9. Wann hat Ihr Kind den ersten kariösen Zahn gehabt?

(0) vor einem Jahr

(1) mit ein bis zwei Jahren

(2) mit zwei bis drei Jahren

(3) später als drei Jahren

10. Benutzt Ihr Kind Zahnseide?

(0) nein

(1) ja

Fluoridanamnese

1. Benutzen Sie fluoridiertes Speisesalz?

(0) nein

(1) manchmal

(2) täglich, aber nicht zu allen Mahlzeiten

(3) immer

2. Bekommt Ihr Kind Fluoridtabletten?

(0) nein

(1) manchmal

(2) oft

(3) täglich

3. Benutzt Ihr Kind fluoridierte Zahnpasta?

(0) nein

(1) einmal täglich

(2) zweimal pro Tag oder öfter

4. Benutzt Ihr Kind Fluoridgel?

(0) nein

(1) manchmal

(2) einmal pro Woche

5. Benutzt Ihr Kind eine Fluoridmundspülung?

(0) nein

(1) manchmal

(2) täglich

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