Assessment of the Status Praesens,
Including the Determining Factors, of Oral
Health in Cystic Fibrosis Homozygotes and
Heterozygotes in Belgium

Johan K. M. Aps
Papa weg ? Papa werken ! Papa tandjes spelen .»

7 A.M., Nathalie Aps, 2 jaar.

To : My parents, my wife Myriam, my daughter Nathalie
     and my parents-in law….. Thank you !
Assessment of the Status Praesens, Including the Determining Factors, of Oral Health in Cystic Fibrosis Homozygotes and Heterozygotes in Belgium

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Dankwoord / Acknowledgements (in Dutch)

Woorden drukken niet altijd volledig uit wat een mens wil zeggen. De non-verbale communicatie verduidelijkt meestal wat men écht bedoelt. Maar ik heb toch en poging gedaan om in dit dankwoord mijn gedachten te verwoorden en de mensen, die mij nauw hebben bijgestaan om dit werk te vervolmaken, oprecht te bedanken.

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Is oral health at risk in people with cystic fibrosis?

Flow cytometry as a new method to quantificy the cellular content of human saliva and its relation to gingivitis.
_Clinica Chimica Acta_ 2002; in press

**Aps J.K.M.**, Delanghe J., Martens L.C.
Salivary electrolytes are compounded with cystic fibrosis transmembrane regulator genotypes.

Caries experience and oral cleanliness in cystic fibrosis homozygotes and heterozygotes.

Mutans Streptococci, Lactobacilli and caries experience in cystic fibrosis homozygotes, heterozygotes and healthy controls.

Oral hygiene habits and oral health in cystic fibrosis.
_European Journal of Paediatric Dentistry; submitted January 2002._

The influence of medication and dietary habits on caries experience and oral cleanliness in cystic fibrosis homozygotes.
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**Aps J.K.M.,** Van Maele G.O.G., Martens L.C. Cariogenic Bacteria and Caries Experience in Cystic Fibrosis homozygotes, heterozygotes and Healthy Controls. 7th World Congress of Preventive Dentistry, Peking (China); 2001; Oral presentation, power point slides. Abstractbook, 7th WCPD congress, page 143-144, abstract 112.

**Aps J.K.M.,** Van Maele G.O.G., Martens L.C. Streptococcus Mutans en Lactobacillus en het cariesvoorkomen bij mucoviscidose homozygoten, heterozygoten en gezonde controlepersonen. 4e Vlaams Congres van het Verbond der Vlaamse Tandartsen, Brugge (Belgium); 2001; Poster presentatie.

**Aps J.K.M.,** Van Maele G.O.G., Martens L.C. Cariogenic Bacteria and Caries Experience in Cystic Fibrosis homozygotes, heterozygotes and Healthy Controls. 45e congres van de Groupement International pour la Recherche Scientifique en Stomatologie et Odontologie, Bruxelles (Belgium); 2001; Oral presentation – Abstractbook, 45e GIRSO congrès, page 14.


Scientific Awards:

Laureate of the Scientific Award of the Belgian Academy of Paediatric Dentistry; Louvain, Belgium. February 26, 1999.

Aps J.K.M., Delanghe J., Martens L.C.
SDS-PAGE of salivary proteins in Cystic Fibrosis: preliminary results.
2nd Congress of the Belgian Academy of Pediatric Dentistry.


Do Dietary Habits Dictate Caries Experience in Cystic Fibrosis Homozygotes?
18th congress of the International Association of Pediatric Dentistry.
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Chapter 1

INTRODUCTION
1.1. Caries Experience and Prevalence

Caries experience is commonly expressed as “DMF-T/-S”. This so called caries index was first introduced by Klein and co-workers in 1938 [Klein et al., 1938]. It was a criterion to validate an individual’s experience with dental decay. The index is composed by three factors; D standing for dental decay, M standing for teeth missing due to caries and F standing for filled teeth. Further, DMF can be expressed on tooth (-T) and on surface (-S) level. This index, which is however worldwide used, is currently under discussion. Especially factor F, which should give an idea of the extend of the experienced decay, is being questioned. The size of a dental restoration is in many cases determined by the dentist and not by the extend of the carious lesion itself. As amalgam used to be the only material of choice for the dentist in the past, the same dentist nowadays has many other possibilities to treat an active carious lesion, with less substance loss for the tooth. Therefore, it is also difficult to compare DMF data from different time eras. Nevertheless, the index remains widely used as it provides a reasonably accurate historical account of changes in the prevalence of caries, as well as in incidence [Vanobbergen, 2001].

Caries prevalence in children, which is epidemiologically determined in cross-sectional and longitudinal studies, has declined the past twenty to thirty years in the Western world [Glass, 1982; Marthaler, 1990; Truin et al., 1994; Elderton, 1994; Marthaler et al., 1996; Petersson and Bratthall, 1996; Vanobbergen et al., 2001a; Vanobbergen, 2001]. It is however striking, that about 10 to 15% of all children now experience 50% of all carious lesions. Figure 1.1. illustrates the skewed distribution to the left. The latter means that these subjects should be regarded as “high caries risk individuals”. This was also found in the Belgian Signal-Tandmobiel® Project, in which this skewed distribution was also observed in 4351 Flemish 7 year olds. Fourty four percent of the 7 year olds in this study were caries
free and 50% of all caries lesions was confined to 15.2% of the children [Vanobbergen, 2001].

Figure 1.1. : Distribution of dental caries (DMF-T) in 7 year olds in Flanders
[J. Vanobbergen, thesis, Ghent 2001]

The WHO goals for the year 2000, a maximum mean DMF-T score of 3 for 12 year olds and a target of 50% of children caries free at the age of 5, have apparently been reached in many West-European countries [Petterson and Bratthall, 1996]. The main reason, being assumed responsible, for this decline of the overall caries prevalence, is the abundant availability of fluorides, more explicitly in toothpaste.
Not only the number of decayed, missing and filled teeth (DMF-T) did decline over the years, but also the extensiveness of dental decay (D-S) did become smaller. The latter can be derived from the extensiveness of the dental restorations (F-S). This can be attributed to two aspects; first of all the better oral hygiene in combination with fluoride containing toothpastes and secondly the less tooth-tissue-destructive restoration techniques in dentistry nowadays (e.g. composites and other adhesive restorative materials) [Cameron and Widmer, 1998; Vanobbergen, 2001].

The above mentioned 15.2% of children who “carry” all caries in Flemish 7 year olds should be the target group in dental prevention. Therefore methods to identify and to reach these “high risk groups” are being explored. From the same study it was observed that the children at high risk for developing dental decay generally may come, on the one hand, from a socio-economically deprived environment (e.g. mother and father unemployed, parents without diploma) and that self esteem plays an important role in a child’s caries experience [Vanobbergen et al., 2001]. On the other hand certain groups of medically compromised and disabled children should not be forgotten as potential high risk individuals; although very few studies are known [Dahllöf and Martens, 2001]. The latter group is assumed to be more prone for dental decay, in part due to the type of their medication intake (e.g. syrups, inhalers, sugar containing medication). In table 1.1. the incidence or prevalence of chronic health conditions in childhood per number of live-births are presented. This table clearly shows that chronic health conditions in children are not rare and that the chance to come across such a patient in a general dental practice is quite large. Literature about the impact of the medical conditions on oral health is rather sparse.

From a study in the south of Sweden it was observed that 8.4% of children aged between 0 and 15 years old suffered from chronic health conditions [Westbom and Kornfält R, 1987]. Among these children, 70% suffered from one condition, while 21% was even affected by two chronic health conditions and in 9% at least 3
<table>
<thead>
<tr>
<th>Disease</th>
<th>Incidence in number of live births</th>
</tr>
</thead>
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<tr>
<td>Mental Retardation</td>
<td>1 to 2 / 100</td>
</tr>
<tr>
<td>Asthma</td>
<td>2 to 15 per 100 children</td>
</tr>
<tr>
<td>Obstructive Sleep Apnea Syndrome</td>
<td>2 per 100 (4 to 5 year olds)</td>
</tr>
<tr>
<td>Attention Deficit Disorder</td>
<td>3 to 5 / 100</td>
</tr>
<tr>
<td>Anxiety Disorders</td>
<td>7 to 15 / 100</td>
</tr>
<tr>
<td>Sickle Cell Anaemia</td>
<td>1 / 600 African-Americans</td>
</tr>
<tr>
<td>Down Syndrome</td>
<td>1 / 660</td>
</tr>
<tr>
<td>Klinefelter Syndrome</td>
<td>1 / 850</td>
</tr>
<tr>
<td>Diabetes type 1</td>
<td>2 per 1000 children (&lt; 18 year olds)</td>
</tr>
<tr>
<td>Congenital Heart Disease</td>
<td>4 to 10 / 100</td>
</tr>
<tr>
<td>Fragile X</td>
<td>1 / 1250 (males); 1 / 2500 (females)</td>
</tr>
<tr>
<td>Cystic Fibrosis</td>
<td>1 to 2500 Caucasians</td>
</tr>
<tr>
<td>Turner Syndrome</td>
<td>1 / 2500</td>
</tr>
<tr>
<td>Congenital Hypothyroidism</td>
<td>1 / 4000</td>
</tr>
<tr>
<td>Haemophilia</td>
<td>1 / 7500 (males)</td>
</tr>
<tr>
<td>Autism</td>
<td>4 to 5 / 10000</td>
</tr>
<tr>
<td>Achondroplasia</td>
<td>1 / 15000</td>
</tr>
<tr>
<td>Phenylketonuria</td>
<td>1 / 10000 to 1 / 15000</td>
</tr>
<tr>
<td>Immotile Cilia Syndrome</td>
<td>1 / 16000</td>
</tr>
<tr>
<td>Kartagener’s Syndrome</td>
<td>1 / 32000</td>
</tr>
<tr>
<td>Galactosaemia</td>
<td>1 / 50000</td>
</tr>
<tr>
<td>Acute Lymphoblastic Leukaemia</td>
<td>4 / 100000 Caucasians</td>
</tr>
<tr>
<td></td>
<td>2.4 / 100000 African-Americans</td>
</tr>
<tr>
<td>Crohn’s Disease</td>
<td>1 to 7 / 10000</td>
</tr>
<tr>
<td>Glycogen Storage Disease</td>
<td>1 / 100000 to 1 / 400000</td>
</tr>
<tr>
<td>Chronic Renal Failure</td>
<td>18 / 100000</td>
</tr>
<tr>
<td>End-stage Renal Disease</td>
<td>3 to 6 / 100000</td>
</tr>
</tbody>
</table>

chronic health conditions were diagnosed. As these children, nowadays, are not always institutionalised, general dentists and especially paediatric dentists are more often confronted with this group of paediatric patients. Moreover, the last two decades, children with a chronic health condition have a great chance to survive their twenties. As a result up-to-date knowledge and know-how of chronic health
conditions has become an important aspect of paediatric dental care. More research is needed to provide more knowledge about the oral health consequences of chronical illnesses. Briefly, chronic health conditions in children can be divided into 5 different patterns [Dahllöf and Martens, 2001] as the expression of the condition may be variable as is the relative consistency or permanence of symptoms or consequences with time (table 1.2.).

**Table 1.2.** Five different patterns of chronic health conditions in children [Dahllöf and Martens, 2001]

<table>
<thead>
<tr>
<th>The pattern is</th>
<th>Per Definition;</th>
<th>Example;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permanent</td>
<td>consistent and unchanging consequences</td>
<td>cerebral palsy</td>
</tr>
<tr>
<td>Recoverable</td>
<td>gradual improvement of sequelae as well as in intensity as in duration</td>
<td>asthma, epilepsy, long-term remission</td>
</tr>
<tr>
<td>Deteriorating</td>
<td>deterioration of the condition can take several years (decades)</td>
<td>cystic fibrosis, muscular dystrophy, aplastic anaemia</td>
</tr>
<tr>
<td>Episodic</td>
<td>alternating periods of disease and relative good health</td>
<td>juvenile rheumatoid arthritis</td>
</tr>
<tr>
<td>No clinical symptoms</td>
<td>no expression of disease</td>
<td>HIV, hypercholesterolaemia</td>
</tr>
</tbody>
</table>

Besides the side-effects of the (chronic) illness, compassionate parents could spoil their medically compromised or disabled child more with sweets and be less strict and demanding in their raising. The latter can also be partly held responsible for children with medically compromised conditions to experience more dental decay than others. These aspects should never be forgotten and once again indicate that caries is a multifactorial disease, which is among many factors also environmentally influenced. Therefore, it is obvious that the prevention of dental caries should be of utmost importance for children and adolescents with medical
compromised conditions, as simple dental problems may severely compromise their medical management [Cameron and Widmer, 1998; Dahllöf and Martens, 2001].

Among medical conditions, currently under discussion and in the sphere of interest of many researchers, are the respiratory disorders.
Chapter 2

Respiratory disorders and oral health

Part of this chapter was published as:
2.1 Asthma

2.1.1. Prevalence and definition

As the number of children with chronic health conditions has increased significantly the last decades, respiratory disorders have gained more and more interest among clinical researchers. Asthma is the most common paediatric chronic respiratory disorder and is defined as a condition associated with reactive airways and reversible airway obstructions which are associated with inflammation. Apparently, till puberty, asthma is more common in boys than in girls, but after puberty the incidence is equal. Geographical differences have also been reported.

The contemporary incidence (table 1.1., chapter 1) of asthma is about 2 to 15% in children. The observed increase in prevalence in the last decades, can not only be explained by environmental conditions and yet the exact cause remains unclear [Light MJ, 2001a]. On the other hand it should be emphasized that this increase in observations can also be caused by a heightened awareness of making the diagnosis and collecting data among physicians.

2.1.2. Oral health and asthma

The oral health consequences for asthma patients, who are under constant aerosol medication, have been studied recently. It is however interesting that both in the north of Europe (Finland and UK) [Mc Derra et al., 1998; Kankaala et al., 1998] as well as in the south (Turkey) [Kargül et al., 1998] asthma studies have been carried out. These studies reported that children with asthma had a significant increased incidence of dental decay, particularly in the permanent dentition. Dental calculus, gingivitis and dental erosions were reported to be significantly more present in this group of children. These features were attributed to their frequent medication intake (aerosols and liquids). Aerosol powders predominantly contain lactose and other sugars as “carriers” for the beta-2-agonists, and as a consequence they may attribute to the observed increased dental decay incidence. A side effect of beta-2-
agonists is a decreased salivary secretion rate, which in its turn may also play a role in the development of dental decay [Ryberg et al., 1987; Ryberg et al., 1990]. Moreover, about up to 80% of the inhaled aerosol medication is retained in the mouth. This figure, however, decreases eightfold when another type of inhaler is used. It needs no further explanation that aerosols taken at night bear a potential caries risk. Furthermore, mouth breathing habits may be held responsible for the increased incidence of gingivitis, while dental calculus was thought to be influenced by increased salivary phosphorus and calcium concentrations in asthma patients.

More recently, the findings of the above mentioned studies were contradicted. A cohort study in New Zealand showed that there was no significant difference between asthmatic children and healthy controls [Meldrum et al., 2001]. The authors agree that certain oral physiological changes may occur due to the disease and the medication, but they emphasize correctly that if a substantial association between asthma and caries would exist, the chances of finding it in their large cohort study were high. Another study, carried out in the USA [Shulman et al., 2001] did not find any differences in caries experience either between asthmatic children and controls. However, when they divided their sample into age categories, the severe asthmatics, aged 4 to 10, had a significant lower caries experience than healthy controls of the same age category. A similar finding was reported recently in a Belgian survey, carried out at the Ghent University [Eloot et al., 2002].

From the above it is clear that results are rather controversial, nevertheless the question remains; “Are patients, who are affected with respiratory problems and obligatory have to take a lot of aerosols, potentially at high risk to develop dental caries?” Besides asthma patients, individuals suffering from cystic fibrosis are also taking lots of aerosols and as a consequence also could run a high caries risk.
2.2. Cystic Fibrosis

2.2.1. What is Cystic Fibrosis?

2.1.1.a. History of the name

“Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die!” [Lewis, 1997]. This quote dates from the seventeenth century and illustrates that cystic fibrosis (CF) is not new. It is believed that CF was brought to Europe several ten thousand years ago, when man was a hunter-gatherer, living in the region of what is now called the Middle East. Ten thousand years ago, man migrated to Europe, to start up living as farmers. On their travelling to the North of Europe, they brought several genetic mutations along, which they mingled with local genes. Consequently today, these mutations can be found all over the world, although the frequency may vary from area to area. Probably, being a CF patient has an advantage in certain regions of the earth and it is believed that it provides a better condition in areas where diarrhoea could be a problem. Apparently CF patients would be less vulnerable to diarrhoea, as the thick mucus lining in their bowel prevents dehydration.

It wasn’t until the twentieth century that science began to unravel the secrets behind CF. Fanconi was the first to report on CF in 1936. Later in 1938, Anderson reported on children dying of lung disease, before their first birthday. She also discovered that not only the lungs were involved in this disease, but also the pancreas. Because of the fibrous scarring of the pancreas tissue the disease was first called “cystic fibrosis of the pancreas”. She also postulated that this disorder was accompanied by not absorbing vitamin A.

In 1945, Farber introduced the name “mucoviscidosis”, as it was discovered that “all” mucus-producing glands were involved as well, causing obstructions in the ducts of these glands [Grundy et al., 1993; Lewis, 1997; Light, 2001b]. Very typical was the observation in New York, 1949, in the summer time, during a heatwave; several CF patients (children) were confronted with massive salt losses.
due to sweating and developed a heat stroke. As recently as 1960, CF patients rarely lived past ten years of age and the therapy for CF was simply dealing with the clinical features of the disease; steatorrhea, respiratory infections (mucous expectorates) and problems to gain body weight. Thanks to improved medical care and knowledge of the disease, CF patients are now able to survive longer than 40 years ago (figure 2.1.) [Mulherin, 1991; FitzSimmons, 1993; Hodson, 1993; Vay Liang, 1993; Webb, 1993; Brewis, 1995; Hamer and Parker, 1996; Lewis, 1997]. Nowadays, the disease is called *cystic fibrosis* in the English-speaking literature, while in Dutch-, French- and German-speaking literature, it is called *mucoviscidose*.

**Figure 2.1.** Increase of survival in cystic fibrosis patients over the years
2.2.1.b. Genetics

Today, CF is known to be the most common lethal autosomal recessive inherited disease in Caucasians which affects both sexes equally and approximately 1 in every 2000 livebirths is a CF baby. About 5% of the Caucasian population is heterozygous for the CFTR (cystic fibrosis transmembrane regulator) gene. The disease is much less frequent in the Black population (1 in every 17,000 livebirths) or in the Asian population (1 in every 100,000 livebirths) [Kunitomo et al., 1991; Tsui et al., 1991; Harris et al., 1991; Aitkin and Fail, 1993; Vay Liang et al., 1993; Hilman, 1997]. As the disease requires two parental genes with the CFTR mutation, both parents are carrier of the disease. In 1988, however, Arthur Beaudet of the Baylor College of Medicine reported a uniparental disomy of CF. A CF patient was born out of a CFTR heterozygous mother and a non-carrier father. However, this exceptional meiosis phenomenon in which the child receives two copies of one parent’s CF bearing chromosome and none from the other parent, should not be forgotten, when examining CF patients [Lewis, 1997]. The responsible allelic sequence is located on the long arm of chromosome 7, at position 7q31. It encompasses a 250 kilobase segment of DNA consisting of 27 axons. The CFTR protein is predicted to be 1480 amino acids long and to have a molecular weight of 160 to 170 kiloDalton. The most frequent CF mutation in Caucasians is a deletion of 3 nucleotides encoding phenylalanine at position 508 (delta F508) in the amino acid sequence. The frequency of this mutation is approximately 70% in the USA and the north of Europe, less than 50% in the south of Europe and only 30% in Ashkenazic families. The highest allelic frequency of delta F508 is found around Denmark (86.6%) while the lowest is found in Turkey (27%) (figure 2.2.). This geographical distribution of the delta F508 mutation is illustrated in figure 2. In essence, the mutation causes a defective chloride channel transport in every exocrine gland of the body.
Figure 2.2. : Distribution of the cystic fibrosis transmembrane regulator genotype ‘delta F508’ in different populations

The most frequent allele causing cystic fibrosis, delta F508, occurs with vastly different frequencies in different populations.

This map indicates the percentage of CF alleles in these nations that are delta F508.

2.2.2. Clinical features of the disease

Fundamentally the disease is caused by a defective sodium and chloride reabsorption mechanism and as a consequence all exocrine glands are affected. The clinical manifestations of this single gene defect are both respiratory and digestive.

The current initial diagnostic tests are focused on the pilocarpine-sweat-test. The higher salt excretion (> 60 mmol/L) when sweating in combination with the following 3 aspects, make the diagnosis of CF complete; (1) chronic obstructive pulmonary disease, (2) exocrine pancreatic insufficiency and (3) a family history of the disease. Finally, nowadays, a genetic diagnosis confirms the observations [Van Nieuw Amerongen, 1988b; Aitken and Fail, 1993; FitzSimmons, 1993; Vay Liang et al., 1993; Brewis et al., 1995].

CF patients are to be medically counselled carefully and an individualized medication programme is indispensable. The respiratory problems involve both upper and lower respiratory tracts. Nasal polyps are common in 10 to 15% of all CF patients [Brewis et al., 1995; Marks and Kissner, 1997]. Obstructive pulmonary disease, with recurrent respiratory infections, caused by *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Staphylococcus aureus*, and acute exacerbations are common in CF. The lower respiratory tract involvement accounts for well over 90% of the morbidity and mortality in CF. Due to extreme mucoid secretions from the pulmonary secretion granules, evacuation of microorganisms is impaired. The latter obligates these patients to take antibiotics (I.V., per os, via aerosols), expectorantia and mucolytica on a regular basis [Bellon and Gilly, 1990; Foucaud et al., 1990; de Montalembert et al., 1991; Hoiby, 1991; Mulherin et al., 1991; Pascal et al., 1992; Wallace et al., 1993; Brewis et al., 1995; Frederiksen et al., 1997]. In table 2.1. some types and names of currently used medication in CF patients are represented with their known side effects. The digestive problems, such as fat and protein malabsorption, result in steatorrhea and failure to thrive and consequently also in leanness. Pancreatic deficiency is the cause, which is in 90%
of all CF patients already present at birth [Herfindal et al., 1988; Aitken and Fail, 1993; Hamer and Parker, 1996]. Pancreatic enzymes, such as Creon® (pancreatine) and Pancrease® (pancrelipasum), have to be taken with every meal and snack.

Sugar-rich in-between-meal snacks and drinks are necessary for the CF patient to gain bodyweight and body-energy. Most patients require supplemental vitamins (A-D-E) as fat malabsorption impairs their uptake via diet. Supplemental vitamin K is only required in cases where, secondary to the disease, liver pathology has developed and blood coagulation is impaired [Sokol et al., 1991; Vay Liang et al., 1993; Durie, 1994; Beker et al., 1997].

A social aspect of CF is that in most male CF patients the reproductive system is involved, frequently resulting in sterility, caused by congenital agenesis of the vas deferens. In CF females there is mainly a decrease in fertility [Harris et al., 1991; Johannesson et al., 1997]. The latter could be interpreted as a disease-self-limiting action by nature itself, in order to prevent spreading. Nevertheless, conception can always take place with or without help from modern current in vitro fertilisation techniques.

2.2.3. Salivary glands and CF

Under healthy conditions, whole human saliva is a mixture of glandular saliva of 3 pair of large glands (Glandula Submandibularis, Glandula Sublingualis and Glandula Parotis), several minor salivary glands on the palate, the cheeks and the mucosal side of the lips, crevicular fluid and a mixture of naso-oro-pharyngeal secretions. In case of CF, all exocrine glands are affected by the CFTR gene and inevitably the salivary glands are also involved. The glandula sublingualis, which is a pure mucous secreting salivary gland, is slightly enlarged under CF conditions. The glandula submandibularis (sero-mucous gland) and the glandula parotis (pure serous gland) are not affected by the disease. Under CF conditions, the salivary
Table 2.1: Currently used medication (in alphabetical order) for airway obstructions (syrups and aerosols) in cystic fibrosis therapy [Source: Compendium 2001 edited by the Associated Pharmaceutical Industry of Belgium]

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Type of medication/ indication</th>
<th>Oral side effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrovent®</td>
<td>anticholinergicum</td>
<td>dry mouth and rasping voice</td>
</tr>
<tr>
<td>Bricanyl®</td>
<td>anti adrenergicum</td>
<td>pH = 3.5, dry mouth</td>
</tr>
<tr>
<td>Bromex®</td>
<td>mucolyticum</td>
<td>! syrup without sugar !</td>
</tr>
<tr>
<td>Clarinase®</td>
<td>antihistaminicum</td>
<td>dry mouth</td>
</tr>
<tr>
<td>Claritine®</td>
<td>antihistaminicum</td>
<td>dry mouth</td>
</tr>
<tr>
<td>Duovent®</td>
<td>anticholinergicum</td>
<td>dry mouth</td>
</tr>
<tr>
<td>Flixotide®</td>
<td>inhalation glucocorticoid</td>
<td>rasping voice + candidosis risk</td>
</tr>
<tr>
<td>Lomudal®</td>
<td>antihistaminicum</td>
<td>dry mouth</td>
</tr>
<tr>
<td>Lysomucit®</td>
<td>mucolyticum / expectorantium</td>
<td>?</td>
</tr>
<tr>
<td>Lysox®</td>
<td>mucolyticum / expectorantium</td>
<td>?</td>
</tr>
<tr>
<td>Mistabron®</td>
<td>mucolyticum / expectorantium</td>
<td>?</td>
</tr>
<tr>
<td>Mucomyst®</td>
<td>mucolyticum / expectorantium</td>
<td>?</td>
</tr>
<tr>
<td>Mucosteine®</td>
<td>mucolyticum / expectorantium</td>
<td>?</td>
</tr>
<tr>
<td>Oxivent®</td>
<td>anticholinergicum</td>
<td>syrup with sugar! dry mouth</td>
</tr>
<tr>
<td>Pulmicort®</td>
<td>anti allergicum</td>
<td>rasping voice and candidosis risk</td>
</tr>
<tr>
<td>Pulmoclase®</td>
<td>mucolyticum</td>
<td>syrup with sugar!</td>
</tr>
<tr>
<td>Pulmozyme®</td>
<td>mucolyticum</td>
<td>laryngitis</td>
</tr>
<tr>
<td>Rhinathiol®</td>
<td>antitussivum</td>
<td>syrup with sugar! pH = 5</td>
</tr>
<tr>
<td>Rhinoebryl®</td>
<td>antihistaminicum</td>
<td>dry mouth</td>
</tr>
<tr>
<td>Serevent®</td>
<td>beta-2-sympaticomimeticum</td>
<td>stimulation of viscous saliva</td>
</tr>
<tr>
<td>Zaditen®</td>
<td>bronchodilatation</td>
<td>dry mouth</td>
</tr>
</tbody>
</table>

consistency becomes more mucous as the percentage of mucous saliva will be higher than under healthy conditions. The composition (both organic and inorganic) of the CF patients’ saliva is also reported to be different than under normal conditions, but no clear data are known [Martinez, 1982; Kinirons, 1983; Van Nieuw Amerongen, 1988a,b,c; Fernald et al., 1990]. Most reports seem to be based on clinical observations and as a consequence should be interpreted with
care. CF patient’s saliva is claimed to have a higher buffer capacity and a higher calcium concentration than that of controls subjects. CF patients would consequently suffer more from calcium plugs resulting in obstructions of the salivary ducts. The increased salivary buffer capacity in combination with the higher salivary calcium concentration would make them more prone for developing calculus [Kinirons, 1983; Van Nieuw Amerongen, 1988a,b]. More recently, a study investigated salivary sodium and chlorine concentrations in CF children (n = 9, mean age 12 ± 3.6 years) [Jiménez-Reyes and Sanchez-Aguirre, 1996] and claimed that they had significantly higher salivary sodium concentrations than healthy controls. They suggested that saliva could be useful in the confirmation of the CF diagnosis. In the mean time, since 1996, no further experiments with CF saliva as diagnostic tool have been published. Probably, because their findings were coincidental and only based on nine subjects with CF. Nevertheless, studies like these are necessary to gather more information on the phenotypic features of CF. They can be the basis for a new more profound study or for a diagnostic technique, which does not require invasive methods.

2.2.4. Cystic fibrosis and oral health

As mentioned in chapter 1, little is known about the impact of disease on oral health in many medically compromised patient groups. Nevertheless, it is obvious that due to the better medical care and medical knowledge about these conditions, patients are surviving longer and are confronted with “new” aspects of their disease. The increased use of aerosols with mucolytica and expectorantia, the increased use of specific antibiotics, administered at the right moment and the correct use of dietary supplements and pancreas enzymes have made it possible for CF patients to survive into their twenties and thirties and some even longer [Mulherin et al., 1991; Webb, 1991; FitzSimmons, 1993; Vay Liang et al., 1993; Brewis et al., 1995; Hamer and Parker, 1996].
Unfortunately, the effect of aerosols on teeth (see chapter 2, 1. Asthma) is being discussed in the literature and it is being considered as a potential high caries risk factor [Kargül et al., 1998; Kankaala et al., 1998; Mc Derra et al., 1998; Dahllöf and Martens, 2001]. The acidity of the aerosols in combination with the carrier (lactose or another sugar) could play an important role in the development of caries. On the other hand the inherent effect of this medication, a decreased salivary flow, should also be emphasized. Especially when the aerosols are used after toothbrushing before going to bed. No data are known for CF patients, but it can be assumed that only little difference in effect of this medication will exist between asthma and CF patients. Medication without sugar should be promoted in medicine in order to decrease the potential caries risk of medication (not only aerosols) intake.

The increased use of antibiotics over the past twenty years has indeed improved the general public’s health. But as every medal has two sides, the widespread use of antibiotics since the 1960s also caused a parallel increase in resistant microorganisms. Clinical resistance to penicillin was already reported in 1965 in Boston, USA. In 1967 the same phenomenon was reported in Australia and ten years later in Africa [Bellon and Gilly, 1990; Foucaud et al., 1990; Appelbaum, 1992; Pascal et al., 1992; Hamer and Parker, 1996]. Factors influencing this emergence of resistance include indiscriminate use of antibiotics, prolonged hospitalizations, increasing numbers of immunocompromised patients and medical progress resulting in increased use of invasive procedures and devices. Resistance can be both obtained direct as well as indirect. “Direct” can be defined as resistance that is being developed to the agent that is used in the antimicrobial therapy, while “indirect” can be defined as resistance being developed through selection of resistant micro-organisms due to an antibiotic treatment or when the environment is “contaminated” with antibiotics (hospital), or via antibacterial agents in agriculture (food) and animal husbandry [Rubinstein, 1999]. The three
most common respiratory tract pathogens, *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella cattarrhalis*, all exhibit high-level resistance to one or a number of agents, including penicillin, ampicillin, erythromycin, tetracycllin and first-generation cephalosporins. *Pseudomonas aeruginosa* and other gram negative bacilli have become increasingly resistant to beta-lactam antibiotics, including imipenem, as well as to fluoroquinolone [Chenoweth and Lynch, 1997; Jones, 1999]. Multidrug resistance, defined as lack of susceptibility to penicillin and at least two other non-ss-lactam classes of antimicrobial drugs, is observed with increasing prevalence in countries all over the world [Appelbaum, 1992; Levy, 1992; Kunin, 1993; Magee et al., 1999; Amyes, 2000].

Dental literature reports that CF patients would gain profit from this abundant use of antibiotics, as it is not imaginary to assume that oral cariogenic microorganisms, such as *Mutans streptococci* and *Lactobacilli*, may virtually disappear from the oral cavity, resulting in less dental decay. This theory was supported in the 1980s by Kinirons in his studies on the oral health of children with CF, in which he discussed the significant lower caries prevalence in CF patients than in healthy controls [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990].

On the other hand, it should be emphasized that, as mentioned above, when resistant oral cariogenic micro-organisms would develop, antibiotics would have an opposite effect and caries would still develop. It is clear that no straight answers can be provided, as no studies are available on oral cariogenic micro flora being affected by oral systemic antibiotics taken on a long term basis.

Recently in 1995, a case-report appeared on 3 adolescent CF homozygote males with a mean DMF-S of 28. The expected low caries incidence was clearly not always the case and this case report supported the hypothesis that CF patients may in time develop a kind of resistant oral microflora, which still can be as cariogenic as the original [Martens and Robberecht, 1995]. Similar findings in 60 children,
over 3 years old, with CF, attending a CF care centre in Manchester (UK) were reported to have a high caries experience [Clarkson and Sood, 1996].

The diet of a CF patient is focused on the gaining of body energy from sucrose rich components, which are to be consumed in-between-meals (drinks and snacks) [Webb, 1991; Vay Liang et al., 1993; Bentur et al., 1996; Marelich and Cross, 1996, Najera-Perez et al., 1997]. This implies that their frequency of eating is considerably high, which is commonly accepted in dentistry as a “high caries risk profile”. The number of sugar-rich-in-between-meal-snacks and drinks are modifying for the rate at which dental plaque and consequently caries will develop [Beck et al., 1988; Hunter, 1988; Mundorff-Shrestha et al., 1994; Van Loveren and van der Weijden, 1996a,b; Palenstein-Helderman et al., 1996; Jensen, 1999]. Consequently, CF patients potentially run a high caries risk. On the other hand, CF patients are also obliged to have extra pancreas supplements with every meal and snack, in order to be able to digest dietary fats. Many children with CF report to open the enzyme capsules and to mix the content in a yogurt drink, instead of swallowing the “large” capsules. The influence of these pancreas enzymes on the oral environment is not known. It may be possible that pancreas enzymes eliminate a large proportion of the oral micro-organisms, resulting in a lower caries experience among CF children [Van Nieuw Amerongen, 1988,]. Once the habit of opening the capsules (which is practised by most of the CF children) is left behind, the pancreas enzymes do not come into contact with the oral cavity any longer and caries experience may still increase. The latter could also explain the high caries incidence in the three adolescent CF patients reported by Martens and Robberecht in 1995.

From the above it can be concluded that CF patients may have a higher caries risk potential, than earlier described in the 1980s. Consequently there is a need for a thorough investigation on this issue.
2.2.5. Pilot study

In order to find out if oral health was really at risk in cystic fibrosis patients, a case-control pilot study was carried out.

2.2.5.1. Material and methods

A sample of 37 CF patients and a similar number of age matched control subjects was selected. Control subjects were selected from the same street, community or school as the CF patients. In this way, socio-economic and demographic factors were matched. All CF patients were sampled from the Belgian Association for the Fight against CF (BAFCF). After mailing twice all CF patients associated with the BAFCF, 5% replied to be interested in participating in this project. The mean age of both groups was 16.3 +/- 8.3 years old. The sample was divided into three age categories (AC): six to twelve year olds (AC-1) representing the primary school age children (15 pairs), thirteen to eighteen year olds (AC-2) representing the secondary school age adolescents (9 pairs) and the older than nineteen year olds (AC-3) representing the adult population (13 pairs). After a signed consent, all subjects were examined by the same trained paediatric dentist, using standardized and widely accepted criteria as recommended by the WHO report on oral health surveys [WHO, 1987] and based on the diagnostic criteria for caries prevalence surveys published by the British Association for the Study of Community Dentistry, BASCD [Pitts et al., 1997]. The investigator (J.A.) was specifically trained at baseline and participated in an in vitro and a clinical calibration exercise according to the guidelines on training and calibration published by the BASCD [Pine et al., 1997].

All subjects were examined under the same conditions either at their homes or at the dental out-patient clinic. Teeth were scored using a WHO/CPITN type E probe, a white light source, cotton rolls and a dental mirror. Decay was recorded at the level of cavitation and the caries experience was expressed by means of the DMF-
T and DMF-S index. The dental care index was expressed as F/D+F (T) and (S). Dental plaque was recorded when visible. This was recorded for every tooth and every surface. Bleeding of the gingiva was recorded when present on gentle probing. This was recorded for every tooth and every tooth surface. All data were immediately stored in a database on a Compac® Armada 1700 computer. The data entry program was prepared by the department of medical informatics and statistics. Appropriate statistical analyses (Wilcoxon matched-pairs signed ranks test and Kruskal-Wallis test) were performed and the level of significance was chosen at $\alpha = 0.05$. The study was approved by the ethical committee of the Faculty of Medicine of the University of Ghent.

2.2.5.2. Results

The investigator’s overall kappa values for both the in vitro and the clinical calibration exercise were respectively 0.84 and 0.76, both standing for an acceptable interreliability with an experienced examiner (L.M.) as benchmark [Bolin et al., 1995].

Thirty five percent of the CF patients ($n = 13$) had no caries experience (DMF = 0) versus 21.6 % for controls ($n = 8$). This difference was not significant between both groups. From Table 2.2. it can be seen that there was no significant difference nor for caries experience (Fig. 2.3.), neither for the dental care index between both samples (Fig. 2.4.).

In Table 2.3. the mean values of the separate DMF factors for each AC are presented. Statistical analysis (Kruskal-Wallis test) of the separate mean DMF-factors between the 3 AC in each sample was conducted.
Table 2.2. Caries Experience (DMF-T and DMF-S) and the Dental Care Index (F/D+F (T) and F/D+F (S)) in Cystic Fibrosis patients versus Controls. (Wilcoxon matched-pairs signed ranks test)

<table>
<thead>
<tr>
<th>Index</th>
<th>Cystic Fibrosis</th>
<th>Control</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF-T</td>
<td>4.35 (5.57)</td>
<td>4.95 (5.04)</td>
<td>0.648</td>
</tr>
<tr>
<td>D-T</td>
<td>0.32 (0.82)</td>
<td>0.54 (1.17)</td>
<td>0.375</td>
</tr>
<tr>
<td>M-T</td>
<td>0.30 (0.94)</td>
<td>0.57 (1.37)</td>
<td>0.231</td>
</tr>
<tr>
<td>F-T</td>
<td>3.78 (4.84)</td>
<td>3.95 (3.96)</td>
<td>0.905</td>
</tr>
<tr>
<td>DMF-S</td>
<td>10.14 (16.96)</td>
<td>13.00 (17.42)</td>
<td>0.291</td>
</tr>
<tr>
<td>D-S</td>
<td>0.43 (1.09)</td>
<td>1.30 (4.65)</td>
<td>0.303</td>
</tr>
<tr>
<td>M-S</td>
<td>1.49 (4.69)</td>
<td>2.84 (6.83)</td>
<td>0.231</td>
</tr>
<tr>
<td>F-S</td>
<td>8.22 (13.17)</td>
<td>8.86 (10.51)</td>
<td>0.583</td>
</tr>
<tr>
<td>F/D+F (T)</td>
<td>0.85 (0.31)</td>
<td>0.86 (0.27)</td>
<td>0.139</td>
</tr>
<tr>
<td>F/D+F (S)</td>
<td>0.86 (0.29)</td>
<td>0.87 (0.28)</td>
<td>0.169</td>
</tr>
</tbody>
</table>

For the CF group (Fig. 2.5.) a significant increase was found for mean DMF-T (P = 0.001) with increasing age. The mean DMF-S was significantly lower in AC2 than in both other AC (P = 0.004). The factor filled, was significantly different between the 3 age categories, both on tooth (P < 0.001) and on surface level (P = 0.002) and increased also with ageing. The separate factors decay and missed between the age categories were not significantly different in the CF sample.

In the control sample (Fig. 2.6.), caries experience (DMF), both on tooth (P < 0.001) and surface level (P < 0.001) also increased significantly with age. These significant differences were confirmed for the separate parameters M and F, as well on tooth level (P = 0.024 and P < 0.001 respectively) as on surface level (P = 0.024 and P < 0.001 respectively). Factor D, however, was significantly lower in AC2 than in both other AC, both on tooth level (P = 0.031) and on surface level (P
= 0.030). With respect to the dental care index (Fig. 2) on tooth level, a significant difference was found with increasing age (P = 0.036) in the CF sample.

**Figure 2.3.** Caries index of the cystic fibrosis group (CF,n=37) versus the Control group (C,n=37)

On surface level, this difference was not significant (P = 0.055). This was not found for the control group. There was no significant difference between CF patients and controls for the number of teeth covered with dental plaque. The same was found, comparing the age categories in both groups. On average, CF patients have less gingival bleeding than controls; this was, however, not significant different (P = 0.067).
**Figure 2.4.** Dental care index per age category (AC: AC1<13y, AC2 = 13-18y and AC3>18y) for the Cystic Fibrosis group (CF,n=37) and the Control group (C,n=37)

**Table 2.3.** Caries Experience (DMF-T and DMF-S) and Dental Care Index (F/D+F (T) and F/D+F (S)) for each age category (AC : AC1 < 13 y, 13y ≤ AC2 ≤ 18y, AC3 > 18y) in the cystic fibrosis (CF-) and control (C-) group. (Wilcoxon matched-pairs signed ranks test)
Figure 2.5. Caries index (DMF-T/-S) for the Cystic Fibrosis group (CF, n=37) as a function of age category (AC: AC1<13y, AC2 = 13-18y and AC3>18y)

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td>N=15</td>
<td>1.80</td>
<td>1.53</td>
<td>0.928</td>
<td>2.22</td>
<td>3.56</td>
<td>0.309</td>
<td>8.77</td>
<td>9.85</td>
</tr>
<tr>
<td>AC2</td>
<td>N=9</td>
<td>0.47</td>
<td>0.40</td>
<td>0.861</td>
<td>0.22</td>
<td>0.00</td>
<td>0.317</td>
<td>0.23</td>
<td>1.08</td>
</tr>
<tr>
<td>AC3</td>
<td>N=13</td>
<td>0.13</td>
<td>0.00</td>
<td>0.655</td>
<td>0.11</td>
<td>0.22</td>
<td>0.655</td>
<td>0.62</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.20</td>
<td>1.07</td>
<td>1.000</td>
<td>1.89</td>
<td>3.33</td>
<td>0.263</td>
<td>8.08</td>
<td>7.69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC1</td>
<td></td>
<td>3.87</td>
<td>3.33</td>
<td>0.694</td>
<td>3.78</td>
<td>6.44</td>
<td>0.312</td>
<td>21.77</td>
<td>28.69</td>
</tr>
<tr>
<td>AC2</td>
<td></td>
<td>0.53</td>
<td>0.47</td>
<td>0.730</td>
<td>0.22</td>
<td>0.00</td>
<td>0.317</td>
<td>0.46</td>
<td>3.15</td>
</tr>
<tr>
<td>AC3</td>
<td></td>
<td>0.67</td>
<td>0.33</td>
<td>0.655</td>
<td>0.56</td>
<td>1.11</td>
<td>0.655</td>
<td>3.08</td>
<td>6.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.67</td>
<td>2.53</td>
<td>0.721</td>
<td>3.00</td>
<td>5.33</td>
<td>0.156</td>
<td>18.23</td>
<td>18.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
<th>CF</th>
<th>C</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFM-T</td>
<td>F/T</td>
<td>0.57</td>
<td>0.72</td>
<td>0.180</td>
<td>0.89</td>
<td>1.00</td>
<td>0.317</td>
<td>0.98</td>
<td>0.88</td>
</tr>
<tr>
<td>DFM-S</td>
<td>F/T</td>
<td>0.60</td>
<td>0.74</td>
<td>0.180</td>
<td>0.92</td>
<td>1.00</td>
<td>0.317</td>
<td>0.97</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Figure 2.5.: Caries index (DMF-T/-S) for the Cystic Fibrosis group (CF, n=37) as a function of age category (AC: AC1<13y, AC2 = 13-18y and AC3>18y)
2.2.5.3. Discussion

This study shows that there are no statistical significant differences in caries experience between CF patients and age matched healthy controls, which is in contradiction with earlier findings [Kinirons, 1983; Kinirons, 1985; Van Nieuw Amerongen, 1988; Kinirons, 1989; Fernald et al., 1990]. On the other hand it has to be stressed that the mean age of the total sample in the present study was 16.3 +/- 8.3 years old, which is older compared to earlier studies.

Nevertheless, in the present AC1, being an age category comparable to these earlier studies, no significant difference was found regarding any parameter of the caries index.
Due to the better medical care and better knowledge, CF patients are now able to survive into their thirties and even older, dependent of the phenotypic expression of their genetic mutation [Hughes and Griffith, 1984; Mulherin et al., 1991; Webb, 1991; FitzSimmons, 1993; Hodson, 1993; Brewis et al., 1995; Hamer and Parker, 1996]. As epidemiological data on oral health in CF adolescents and adults are lacking, the present report is the first of its kind. By dividing the samples in 3 age categories, we were able to compare CF patients from childhood, through adolescence into adulthood. As no significant differences were found in caries experience between CF patients and controls and regarding the presence of potential caries risk factors, three hypotheses can be postulated.

(i) As there are probably geographical differences, the earlier findings by Kinirons [Kinirons, 1983; Kinirons, 1985, Kinirons, 1989] are specifically representable for CF patients in Northern Ireland. Other environmental influences such as: drinking water fluoridation, social economical status differences and the availability of community dentistry centres where children are seen on a regular basis from age zero, will contribute to regional differences.

(ii) As the reported DMF-T indices in young and adolescents subjects (Fig. 2.4.) perfectly fit within the WHO objectives for the year 2000 [Nithila et al., 1998], it is possible that as a consequence of a broader use of antibiotics and aerosols, the “healthy” control subjects have become more similar to the CF population. The latter can explain why in none of the age categories significant differences in caries experience were found.

(iii) A third hypothesis could be that medicine in a particular era plays an important role. In the nineteen eighties, the medical care for CF patients was different from today’s [Herfindal et al., 1988; Bellon and Gilly, 1990; Foucaud et al., 1990; Pascal et al., 1992; Brewis et al., 1995; Hamer and
Parker, 1996; Marks and Kissner, 1997]. Due to this better medical attention and the individualized medication programmes, it could be assumed that CF patients also obtained better oral health than 20 years ago and subsequently became in a way more “similar” to control subjects.

When going into more detail, it is remarkable that the number of caries experience free individuals is not significant different between both groups. This means that CF patients do not seem to run a higher caries risk than controls. Neither of the age categories is significant different between CF patients and controls. This contradicts clearly earlier findings [Kinirons, 1983, Kinirons, 1985, Kinirons, 1989, Fernald et al., 1990]. The mean age of the caries experience free CF patients was 11.6 years old and for the controls 13.4 years old, which wasn’t significant different either.

DMF-T increased significantly with ageing in the CF sample. The same holds for the control group’s DMF-T and DMF-S. In the CF sample, DMF-S in AC2 was lower than in AC1 and AC3. This is due to the fact that in the CF group, factor “decay” and “missed” are lower in AC2 than in both other age categories, although not significantly different. Only the number of restored teeth and surfaces increased significantly with age. This implies that not only the number of restorations increase with age, but also the dimension of the restorations. This confirms the more recent findings [Martens and Robberecht, 1995] reporting a higher caries prevalence in 3 CF adolescents, with a mean DMF-S of 29. It was suggested that CF patients might have a lower caries experience at young age, but that the incidence of caries increased during ageing, due to intrinsic factors of the disease.

In the control sample both factors “missed and filled” increased significantly with ageing, while factor “decay” was significantly the lowest in AC2. Actually no decay was present in AC2. This can probably be explained by the fact that the healthy controls at the time of examination all had a permanent dentition, which
had just erupted, or were in the middle of exfoliation of the deciduous canines and molars. The opportunity for caries to be present in this transitional period is rather low.

The dental care index, expressed as F/D+F (T) and (S), wasn’t significantly different between both samples. This means that both groups are equally “well cared for” dentally. The dental care index in controls did not increase significantly with ageing, nor on tooth level, nor on surface level. In CF patients, dental care index increased significantly on tooth level, and only borderline not significantly on surface level. This means that control subjects seem to attend to dental visits at an earlier age than CF patients. This implies that controls in a certain way, maintain a “constant” dental care index, while in CF patients, dental visits are probably postponed as their parents are confronted with other “important aspects of their children’s health”. Dental care would probably be the last of their worries, as this is not considered as life-threatening. It is comprehensible that once CF patients survived their teenage years, their parents are less reluctant to attend to dental visits. Orthodontic problems could also be one of the main reasons why CF parents would suddenly submit their CF child to dental care, consequently resulting into better conservative and preventive dental care.

It remains however remarkable that although they seem to attend to dental visits later in life, they do not have a significant higher caries experience than controls. This supports the hypothesis [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989] that CF patients are in a way protected, although they have frequent antibiotic (sugar-containing) syrups, daily aerosols with mucolytica and expectorantia, an increased salivary viscosity and frequent in between-meal-snacks. All the latter are well known as caries risk factors, which should render CF patients more susceptible to dental decay. Probably there are intrinsic factors of the CF disease, which protect CF patients. As frequent antibiotics cause resistant micro-organisms, the earlier proposed hypothesis [Kinirons, 1985], that CF patients are protected by their
antibiotic treatments, should be disproved. Aerosols dry out the oral cavity and mostly have an acidic pH. This also bears a potential caries risk, which is recently shown in asthmatic patients [Kargül et al., 1998; Kankaala et al., 1998]. CF patients have to use this type of medication sometimes several times a day [Bellon and Gilly, 1990; Foucaud et al., 1990; Pascal et al., 1990; Brewis et al., 1995; Hamer and Parker, 1996; Marks and Kissner, 1997]. CF patients also need to have several in-between-meal-snacks, in order to provide them of the necessary body-energy [Hughes and Griffith, 1984; Tsui, 1991; Grundy et al., 1993; Vay Liang et al., 1993; Bentur et al., 1996]. These are mainly sugar-rich snacks and drinks, as they are unable to digest fats. In combination with every meal or snack, CF patients also need to take additional pancreas enzymes. Sometimes as much as 80 capsules a day! Most of the small children reported to open the capsules and mix the content with a yoghurt or other drink. The effect of the enzymes in the oral cavity on the oral bacterial flora is not known. Once they get older, after the age of 11 or 12, most of them report to be able to swallow the large capsules. Opening the capsules probably destroys micro-organisms, and consequently bears a protective aspect with regard to dental decay.

With regard to the oral cleanliness, it is expected that brushing habits are disorganized, especially in the morning, as mucus is blocking their airways and gagflexes are stimulated. Similarly, during hospitalized antibiotic treatments and periods of severe health problems, oral cleanliness can also be expected to be impaired. No differences were found, however, between CF patients and controls for the amount of plaque. There wasn’t a significant difference between the age categories in both samples. CF patients had less, although not statistically significant for AC1 and AC2, gingival bleeding sites than controls. This could mean, in contrast to the above mentioned suggestion, that CF adults brush more carefully than healthy adults which is probably related to the fact that they are more conscious of their health and hygiene than controls or that other intrinsic
protective mechanisms are involved. The fact that they survived into their twenties is probably for most CF patients an extra motivation to take good care of their health, as they are well aware of the potential lethal risks they run when encountering a bacterial infection.

2.2.5.4. Conclusion

There are no significant differences in caries experience, dental care index and dental plaque between CF patients and healthy control subjects. CF adults have significantly less gingival bleeding sites than adult healthy controls. As a result of these findings, former literature [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990] on oral health and CF is contradicted and more recent suggestions [Martens and Robberecht, 1995; Clarkson and Sood, 1996] are confirmed. It is clear that still more refined analyses are necessary in order to declare the phenomenon of having a medically compromised group which is intrinsically at risk for caries development and which experiences no more caries than healthy controls. For this a large, perhaps multicentre study in depth regarding oral hygiene, dietary habits, salivary properties, medication intake etc. should be planned.
Chapter 3

Aims of the study
3.1. Introduction

Up to now, no oro-dental data whatsoever were available on CF patients in Belgium. Moreover, within the international literature only data about North Irish children were reported. As a consequence a need was felt to investigate differences in oral health between CF patients, CF heterozygotes and healthy controls in Belgium and to define the determinants of oral health within the three groups.

As CF heterozygotes can now be distinguished from the general population through genetic testing, they should be considered as a separate group and as a consequence they would not be mingled with control subjects as was inevitably done in the past.

The null hypothesis of this study is that CF patients are potentially at risk for oral health and that saliva can be used for its determination.

3.2. Caries experience and oral cleanliness

As only data on caries experience in CF children were available in the literature [Kinirons, 1983; Kinirons 1985; Kinirons, 1989] a need was felt to examine Belgian CF children, as well as adolescents and adults. Both the latter groups were now “available”, compared to the 1980s when not that many CF children survived their teenage years.

Caries experience (figure 3.1.) is a valuable parameter for oral health as it reflects different factors which are of influence; oral hygiene habits, dietary habits, environmental factors, bacterial constitution of the oral cavity and host factors. The impact of every factor is, however, inter-individually different. Caries experience also provides a view into the past, already explained in chapter 1, while oral cleanliness on the other hand, another valuable parameter for oral health, being defined as presence or absence of plaque, calculus and gingival bleeding, are “actual” facts, tells something about the present attitudes of a subject.
3.3. Oral micro-organisms

*Mutans streptococci* and *Lactobacillus* are both oral micro-organisms, which belong to the commensal oral flora, but which are considered in the literature [Larmas, 1985; Loesche, 1986; De Soet et al., 1989; Van Houte, 1993; Van Houte, 1994; Kohler et al., 1995; Loesche et al., 1995; Roeters et al., 1995] as indicative...
for caries experience, if present in large amounts ($\geq 10^5$ CFU/ml). Both microorganisms can be cultivated in laboratory environment on agar plates, but commercial, well described in literature, chair-side-tests are also available (Vivadent, CRT® Bacteria and CRT® Buffer) nowadays [Rogosa et al, 1951; Larmas, 1975; Kneist and Heinrich-Weltzien, 1997; Kneist et al., 1999a,b; Twetman et al., 1999]. Mutans streptococci are considered as the “initial” colonizers in caries active individuals, as they generally consume lots of sugars, while Lactobacilli are known to colonize the oral cavity at a later stage, when cavitation is present. Lactobacilli apparently prefer niches to hide in and as a consequence, poorly fitting dental restorations can also be the reason for them to flourish [Marsh and Martin, 1999a,b].

CF patients obligatory consume lots of sugar rich in-between-meal snacks and drinks, which could result in a high Mutans streptococci count, therefore an investigation to study the differences in bacterial counts (Mutans streptococci and Lactobacilli) between CF homozygotes, CF heterzygotes and healthy controls was considered important and opportune.

### 3.4. Medication influence

Certain types of medication are known to influence salivary composition and properties [Van Nieuw Amerongen, 1988c]. CF patients take expectorantia, mucolytica, antibiotics and pancreas enzymes on a daily basis (per os, I.V. and aerosol). The effect of the daily medication of CF patients, however, on their orodental habitat is not known. Therefore a study was needed to investigate the effect on caries experience of opening the capsules with pancreas enzymes, while the effects of the different aerosol medications and antibiotics in CF therapy had to be investigated with oral cleanliness and caries experience as response variables within the CF patients in this study.
3.5. Dietary habits

Dietary habits can also provide a window at an individual’s caries experience and oral cleanliness, as it is well illustrated in literature that sugar rich foods and drinks can be responsible for large amounts of plaque and consequently for caries [Grindefjord et al., 1993; Holbrook et al., 1993; Leverett et al., 1993; Verrips et al., 1993; Grindefjord et al., 1995; Grindefjord et al., 1996; Van Loveren and Van der Weijden, 1996; Palenstein-Helderman et al., 1996; Al Ghanim et al., 1998].

Dietary habits in case of CF patients is even more interesting to investigate as CF patients obligatory consume many sugar rich in-between-meals and drinks. The latter implies that theoretically CF homozygotes potentially run a high caries risk. All the more, because literature on CF patients [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989] claimed that CF children had significantly less caries experience than others, while they behaved like a group at high risk to develop caries, the study had to involve an investigation on the impact of this determinant of oral health in the three groups. Differences in dietary habits could provide an explanation for possible differences in oral health.

3.6. Oral hygiene habits

A subject’s oral hygiene habits can determine in great extent that same individual’s oral cleanliness and caries experience [Van Loveren and Van der Weijden, 1996a; Vanobbergen et al., 2001b; Vanobbergen, 2001].

Therefore an investigation on the differences in oral hygiene habits, which could be held responsible for differences in oral cleanliness or caries experience between the three groups and within the groups was needed. It should however, be emphasized that oral hygiene habits can have changed in time. This implies that current oral hygiene habits will mainly reflect current oral health and oral cleanliness in particular, and to a lesser extent past caries experience. As a consequence, it can be expected that oral hygiene habits will correlate more with
presence or absence of plaque, calculus and gingival bleeding, than with DMF-T/-S.

3.7. Salivary electrolytes

Calcium, bicarbonate, phosphate and sodium play an important role in re- and demineralization of dental enamel [Driessens and Verbeeck, 1990]. In the line of factors that determine oral health, salivary electrolytes should not be forgotten, although little cause-result evidence has been found between dental caries and salivary ions [Van Nieuw Amerongen, 1988a]. However, as CF is a disease characterized by a malfunction in sodium and chlorine exchange through cell membranes of exocrine glands, salivary electrolytes could play an important role in the expression of the different phenotypes of CF and in the prevalence of dental decay in this group of medically compromised [Van Nieuw Amerongen, 1988b]. Secondly, it is interesting to investigate whether differences in salivary electrolyte concentrations exist between CF heterozygotes and healthy controls. Perhaps CF heterozygotes can be identified through salivary electrolyte alterations.

3.8. Salivary proteins

The altered salivary visco-elastic properties of patients with CF is caused by the increased proportion of saliva (viscous) excreted by the Glandula sublingualis; mucins (acidic) and other proteins, binding water molecules, can partly be held responsible. Total salivary protein concentration is reported to be twofold in CF homozygotes [Van Nieuw Amerongen, 1988a,b]. However, nothing is known about CF heterozygotes. Consequently, it can be of importance to determine differences in salivary protein composition and total salivary protein concentration between CF homozygotes, CF heterozygotes and healthy controls.
3.10. Aims of the study

According to the *null hypothesis*, which states that CF homozygotes are at risk for oral health, and that saliva can be used for its determination, the following aims of the study can be postulated:

1. Determination of the caries experience and oral cleanliness of CF homozygotes, CF heterozygotes and healthy controls.

2. Quantification of the oral bacterial flora with respect to caries in CF homozygotes, CF heterozygotes and healthy control subjects.

3. Assessment of the impact of medication, dietary habits and influence of oral hygiene habits on oral health in CF homozygotes, CF heterozygotes and control subjects.

4. Evaluation of salivary electrolyte concentration differences and differences in salivary proteins among CF homozygotes and between CF homozygotes, CF heterozygotes and healthy individuals.

5. Assessing the possibility to use human saliva as a diagnostic tool for the determination of oral health.
Chapter 4

Material and methods
4.1. Patient selection

CF patients and CF heterozygotes were selected via the Belgian Association for the Fight against Mucoviscidosis. From a mailing (anno 1998) to about 700 CF families, 7% replied to be interested to participate in this study. According to the mailing replies, the main reason for not willing to participate was the appropriate fear to get infected with *Pseudomonas*. The second most common excuse was “distance”, although it was suggested that investigations could be carried out at home if required. Finally, in 1999, visits were started, with three CF patients less than planned. Unfortunately, in the mean time, three people with CF had died from the disease. A total of 50 CF homozygotes were seen, either at their homes or in the dental out-patient clinic at the Ghent University Hospital. A drop out of 8 patients was observed due to insufficient volume of saliva sample or due to patient’s moving, resulting in a full documented study group of 42 CF homozygotes. Their mean age was 16.2 ± 8.1 years.

A total of 48 CF heterozygotes were involved in the study. CF heterozygotes, were defined as individuals which were genetically proven to be CF heterozygous for the CFTR gene, Consequently, they were all related to the CF homozygotes in this study and as this group consisted of parents, brothers and sisters of CF homozygotes, their mean age was considerably higher; 29.5 ± 15.9 years.

Healthy individuals (n = 62), being people without cardiovascular, genito-urinary, endocrine, hematological or infectious diseases, were recruited either from the CF homozygotes environment (e.g. school, street or village) or from the Ghent University Hospital dental out-patient clinic. The mean age of the control group was 19.9 ± 11.5 years old.

Prior to participation, all participants signed an informed consent and the study was approved (20/03/1997, number 97/71) by the Ghent University Hospital’s ethical committee. The study was financially supported by the Fund for Scientific Research Flanders (FWO grant: 3.0014.98).
4.2. Caries experience and oral cleanliness

4.2.1. Caries experience

All subjects were examined by the same trained paediatric dentist (J.A.), using standardized and widely accepted criteria as recommended by the WHO report on oral health surveys [WHO, 1987 and WHO, 1997] and based on the diagnostic criteria for caries prevalence surveys published by the British Association for the Study of Community Dentistry, BASCD [Pitts et al., 1997]. The investigator (J.A.) was specifically trained at baseline and participated both in an in vitro and in a clinical calibration exercise according to the guidelines on training and calibration published by the BASCD [Pine et al., 1997]. The investigator’s overall kappa scores for the in vitro and in vivo calibration exercise were respectively 0.84 and 0.76. Both figures stand for an acceptable interreliability with an experienced examiner (L.M.) as benchmark [Bolin et al., 1995]. All subjects were examined under the same conditions either at their homes or at the Ghent University dental out-patient clinic. The home visits were necessary, as most CF patients were appropriately afraid to get infected with *Pseudomonas aeruginosa*. Teeth were scored using a WHO/CPITN type E probe, a white light source, cotton rolls and a dental mirror. Decay was recorded at the level of cavitation and the caries experience was expressed by means of the DMF-T and DMF-S index. The dental care index was expressed as F/D+F (T) and (S). No radiographs were taken.

4.2.2. Dental plaque and dental calculus

As the purpose of this study was to obtain data on the presence and not on the amount of dental plaque and calculus per tooth or per surface (with exception of the occlusal surface), and as in most cases subjects were examined at home, a fast and easy recording technique was considered most appropriate. In order to compare data from the literature, all teeth, except the third molars were involved. Visual presence of plaque and calculus were scored "1" and absence was scored as "0".
In case of doubt, a WHO/CPITN type E probe [http://www.whocollab.od.mah.se] was used for verification. Consequently the presence of dental plaque and calculus was recorded as a numerical value, both on tooth and on surface level.

4.2.3. Gingival bleeding

The gingival status was recorded after assessing caries experience, dental calculus and dental plaque. A simplified version of the Loe gingival index (GI) [Löe, 1967] was used; score "0" was absence of bleeding after gentle probing with a WHO/CPITN type E probe [http://www.whocollab.od.mah.se] and scores 1 to 3 were converted into one score (score "1") corresponding to bleeding after gentle probing. This was recorded for every tooth present (apart from the third molars) and for the respective surfaces. For the reasons mentioned above, as it was not the aim of the study to investigate the ‘severeness’ of the gingival status, no extensive bleeding index notation was considered necessary.

4.3. Probing subject’s habits

4.3.1. Interview

The technique of interviewing was preferred over a written questionnaire which was filled in by the participants themselves for two important reasons. Primo to avoid unanswered questions and secundo to avoid biassed information, which can be expected from the kind of questions asked in a survey. It is commonly accepted that a questionnaire, if not correctly assembled, can bias answers, as people unavoidedly change their answers. The prejudice that for instance a dentist is asking questions about oral hygiene habits, might push subjects to give “false” answers, which do not stroke with their usual habits.

All interviews, about 30 minutes each, were taken by the same investigator (J.A.). Detailed information was obtained about every subject’s dietary habits, oral hygiene habits and medication use. The interview was based on a questionnaire,
which was used with success in previous studies and of which the validity was shown. Regarding reliability, the investigator considered it acceptable that possible ‘noise’ could lead to an equal shift of the results, but as statistical models were used with a dichotomised outcome and explanatory variables, this was to be minimised. Moreover, the investigator had obtained experience in the past, working with this questionnaire, during a collaborative study (Children’s Dental Health in Europe, coordinated by Dr. A.K. Bolin) [Rise et al., 1991a; Rise et al., 1991b; Goldbohm et al., 1994; Osler and Heitman, 1996; Bolin et al., 1997a,b; Vanobbergen, 2001].

4.3.2. Oral hygiene habits

The following items on oral hygiene measures were discussed via an interview during which all answers were dichotomized as “yes” or “no”

- I brush less than twice a day / I brush at least twice a day
- I do not brush my teeth in the morning / I brush my teeth in the morning
- I do not brush my teeth in the evening / I brush my teeth in the evening
- I use at least a pea size of toothpaste / I use at least one centimetre of toothpaste
- I do not use a fluoride containing toothpaste / I use a fluoride containing toothpaste
- I use a regular toothbrush / I use an electrical toothbrush
- I never use a fluoride containing mouthrinse / I use a fluoride containing mouthrinse
- I never had fluoride supplements (drops or tablets) / I had fluoride supplements
- I never use a chlorhexidine containing mouthrinse / I use a chlorhexidine containing mouthrinse
4.3.3. Dietary habits

The composition of every meal and snack was recorded, the time of the day when was eaten and drunk and how and where this was carried out. The questionnaire consisted of questions which could be answered with yes or no (e.g. “do you have breakfast every day?”) and questions which had to be answered more into detail (e.g. “what do you drink with your breakfast?”). Subsequently the investigator (J.A.) listed the answers into categories. Drinks were divided into 4 categories, being; “no drinks, sweetened drinks (e.g. fruit juices, soft drinks, coffee with sugar), non sweetened drinks (e.g. black coffee, plain tea, water) and dairy drinks (e.g. milk, yogurt, coffee with milk)”. Foods were divided into 3 categories, being; ”a hot meal, a sandwich-like meal with a sweet filling (e.g. marmalade) and a non-sweet filling (e.g. meat, cheese, vegetables, salads)”. The desserts were categorized into 4 groups, being; “no dessert, a sweet dessert (e.g. pie, candy bar), fruit and a non-sweet dessert (e.g. cheese)”. Snacks were divided into drinks and food, being; “no drink, a sweetened drink (e.g. fruit juices, soft drinks, coffee with sugar), a non-sweetened drink (e.g. plain tea or coffee), dairy drinks (milk, yogurt, tea with milk), a sweet snack (e.g. candy bar, cookies), fruit and non-sweet snacks (e.g. a cheese or ham sandwich)”.

4.3.4. Medication

Every subject was asked whether medication was used on a regular basis. This could include anticonceptiva as well as expectorantia. The number of intakes per day, the way of taking medication (e.g. aerosol, syrup or intravenously) and the type of medication (e.g. antibiotics, mucolytica or anticholinergica) were recorded meticulously for every subject in this study. For the CF homozygotes, separate
questions about how (e.g. open or closed capsules or opened in a yoghurt drink) pancreatic enzymes were taken, were also included in the interview.

4.4. Saliva collection for analytical purposes

Paraffin stimulated whole saliva was collected in a sterile plastic receptacle. All subjects were in an up-right position at the time of collection. As salivary electrolytes concentrations vary during the day, due to the circadian rythm [Van Nieuw Amerongen, 1988a], samples were collected between 9 a.m. and 4 p.m. in the presence of the same investigator (J.A.). All samples were transported at room temperature almost immediately to the Ghent University Hospital, where their analysis took place and the volume of saliva was devided into several smaller recipients for further analysis. These samples were subsequently stored in a controlled freezer at −17°C.

4.5. Mutans streptococci and Lactobacilli counts

CRT® Bacteria (Vivadent®, Schaan, Liechtenstein) was chosen as a commercial bacterial colony counting test kit, as it is simple to perform and provides the opportunity to evaluate both Mutans streptococci (MS) and Lactobacilli (LB) on the same two-sided dip-slide [Rogosa et al., 1951; Gold et al., 1973; Larmas, 1975; Kneist et al., 1999a,b]. The CRT® Bacteria test-results are expressed as a low (< 10^5 CFU) or a high (≥ 10^5 CFU) bacterial count. Chair-side tests, such as CRT® Bacteria and Dentocult® (Orion Diagnostica®, Finland) have been extensively described in literature as being reliable and easy to use clinically to assess caries risk in populations [Twetman et al., 1994; Kneist and Heinrich-Weltzien, 1997; Kneist et al., 1998; Kneist et al., 1999a,b; Twetman et al., 1999]. Dipslides were inoculated, incubated and evaluated according to the manufacturer’s guidelines.

The investigator (J.A.) was trained at baseline in evaluating the CRT Bacteria commercial test results. Subsequently the same investigator evaluated a total of 40
samples twice at an interval of 8 hours. This was necessary as no major changes of the agar plates was acceptable for intra-examiner reliability assessment. The kappa value was 0.71, standing for an acceptable intra-examiner reliability.

4.6. Salivary electrolytes

Prior to chemical analysis, each sample was vortexed for at least 30 seconds and subsequently 3 milliliter was centrifuged for 10 minutes at 3000 rpm at roomtemperature. Subsequently the supernatant was used for chemical electrolyte analysis (respectively calcium, bicarbonate, phosphate, potassium, sodium and chloride concentrations). Salivary osmolarity was also determined.

Measurement of the total calcium concentration was performed by a colorimetric method and inorganic phosphate was quantified by means of a molybdate complexing reaction method. Both procedures were carried out by means of a testkit provided by Roche® (Mannheim, Germany).

The bicarbonate concentration was determined enzymatically using commercial reagents from BioMérieux® (Biomérieux, Marcy-les-Etoiles, France).

Potassium, sodium and chloride were automatically measured using indirect potentiometry on the Hitachi 747® analyzer (model 100) (Tokyo, Japan).

Salivary osmolarity was measured with the “Advanced cryomatic osmometer®” (model 3C2, Advanced Instruments) (Needham Heights, Massachusetts, USA).

4.7. Salivary proteins

4.7.1. Total protein concentration

Two milliliter of each well vortexed saliva sample was centrifuged at 3000 rpm for 10 minutes. Subsequently the supernatant was pipeted and the total protein content was determined automatically on a Hitachi® 911 automatic analyzer (Tokyo, Japan). This machine uses the pyrogallol-red molybdate complexing
method [Van Ingen, 1990]. Photospectrometry is carried out at a wavelength of 600 nm. The result is expressed as gram total protein per litre saliva.

4.7.2. Sialic acid concentration

Sialic acid (nitrogen-acetylneuramine) was determined on a Hitachi® 911 automatic analyzer (Tokyo, Japan) with help from a Roche® test package. By means of neuraminidase, the sialic group was separated from its glycoprotein. Subsequently nitrogen-acetylneuraminic acid is converted into pyruvate and nitrogen-acetylmannosamine. In a third phase, pyruvate is oxygenated respectively into carbon dioxide, hydrogen peroxide, and acetyl phosphate. By means of the Roche® test package the hydrogen peroxide finally is converted into a red pigmented fluid, which is subsequently measured by photospectrometry (wavelength 550 nm). The peroxide is a measure for the concentration of free nitrogen-acetylneuramine. The concentration is expressed as milligrams sialic acid per litre saliva.

4.7.3. Electrophoresis

4.7.3.1. Molecular mass differentiation

Electrophoresis, in general, is the movement of particles in an electrical field. It is one of the most efficient analytical tools in modern clinical biochemistry to separate the different monomers of a polymer. Saliva can be considered as a polymer and as a consequence, the individual monomers, the salivary proteins, can be identified on basis of their molecular mass (unit = Dalton) by means of “sodium dodecyl sulphate poly acrylamide gel electrophoresis” or SDS-PAGE for short.

4.7.3.2. SDS-PAGE procedure

Three millilitre of each saliva sample was ultracentrifuged (Beckman® L-2) at 13000 rpm for 30 minutes at 10°C. Then the supernatant was frozen at −17°C and
subsequently lyophilized at -70°C (Virtis® Gardiner, NY 12525, USA) for at least 8 hours until only powder was left in the glass bulb. The lyophilized saliva was thereupon diluted in 250 µL sample buffer (pH = 6.8; 3.8 ml distilled water, 1.0 ml 0.5 M Tris-HCl, 1.6 ml 10% SDS, 0.8 ml glycerol, 0.4 ml 1% brominephenol blue (Merck®)) and vortexed. Only 50 µL of this solution was used for further procedure and 10 µL neuraminidase was added. The solution was incubated for 2 hours at 37°C. The latter was necessary to separate the sialic acid groups from the salivary glycoproteins.

In the meantime the electrophoresis buffer solution (pH = 8.3; 9 g of Tris alkaline + 43.2 g of glycine + SDS + diluted with distilled water to a volume of 600 ml) was prepared and stored at −4°C. SDS-PAGE 4-15% Tris-HCl-gradient gels (BIO-RAD® Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547, USA), were mounted in the appropriate Mini-Protean II® electrophoresis cell (BIO-RAD®).

Prior to application of the samples in the gel indentations, the electrophoresis buffer solution is poured into the inner cooling core of the electrophoresis chamber and checked for leakage. Subsequently the outer core was filled and 10 µL samples were applicated in the indented application sites of the gradient gel.

SDS-PAGE was performed at a constant voltage of 200 Volts for 50 minutes. The progression of the electrophoresis was easy to follow because of the samples’ blue color.

After 50 minutes, the gels were removed from the electrophoresis chamber and put in a Coomassie Brilliant Blue R250 solution (LKB Bromma®, a 1g/L dilution in 40% methanol and 10% acetic acid) for 30 minutes. Subsequently the gels were decolorized several times in a solution with 40% methanol and 10% acetic acid until only the blue and purple protein fractions were visible.
4.7.4. Determination of the molecular masses and identification

By means of a standardized protein mix (SDS-PAGE Standards, broad 200 µl, order number 161-031), as provided by BIO-RAD®, the molecular masses of three particular protein fractions were determined via linear regression analysis.

4.8. Data processing and statistics

All data from either the clinical examination or the interview were immediately stored in an automated database (Access file, © Microsoft Corporation) on a Compaq Armada 1700 computer.

Statistics were performed either by means of the MedCalc® statistical programm for biomedical research (MedCalc, Mariakerke, Belgium) or by means of the SPSS programm at the Department of Medical Informatics and Stastistics, RAMIT (Research in Advanced Medical Informatics and Telematics) at the Ghent University Hospital, by Dr. Georges Van Maele.
Chapter 5

Caries experience and oral cleanliness in cystic fibrosis homozygotes, heterozygotes and healthy controls

This chapter was published as:
ABSTRACT

In the present study caries experience (DMF) and oral cleanliness (plaque, calculus and gingival bleeding) of CF homozygotes (n = 42), CF heterozygotes (n = 48) and healthy controls (n = 62) were recorded and statistically analysed. CF homozygotes showed a significant lower caries experience (DMF-T) than both controls (P < 0.001) and CF heterozygotes (P= 0.011). They also had significantly less gingival bleeding sites than controls (P = 0.014) and CF heterozygotes (P = 0.019). CF heterozygotes showed significantly more extensive restorations than CF homozygotes (P = 0.015) and significantly more missing teeth than controls (P = 0.008), while the latter had significantly more missing teeth than CF homozygotes (P < 0.001). Although CF homozygotes potentially run a high caries risk due to their essential sugar rich diet, they did not show a significantly higher caries experience. Their better gingival health and lower caries experience may be attributed to medication use (antibiotics) and certain still, as yet unidentified, intrinsic salivary mechanisms.
5.1. Introduction

Cystic fibrosis (CF) is the most common lethal recessive inherited disease in Caucasians. Around 1 in every 2000 live newborns has CF and about 4 to 5% of Caucasians are heterozygous for the cystic fibrosis transmembrane regulator (CFTR) gene. In essence, the deletion causes increased mucous secretions in every exocrine gland, including the salivary glands (the sublingual glands in particular). CF patients need many in-between-meal-snacks and sugar-rich drinks to provide them with the necessary body energy. This diet is thought to carry a high cariogenic potential. A more detailed description of the disease and useful references were published elsewhere [Martens et al., 2001].

Literature on CF and oral health is rather scarce. Reports from the 1980’s stated that CF homozygotes (all children) had a lower caries experience than healthy controls and a higher tendency to form dental calculus [Kinirons, 1983 ; Kinirons, 1985 ; Kinirons, 1989 ; Fernald et al., 1990]. While most studies deal with younger children, a recent study [Martens et al., 2001] compared the caries experience between CF homozygotes and controls, comprising a wider age range. This type of study only became possible as CF patients survived into their teenage years as a consequence of better medical care and knowledge [FitzSimmons, 1993]. It did, however, not confirm the lower caries experience in CF patients, as reported in the literature [Kinirons, 1983 ; Kinirons, 1985 ; Kinirons, 1989 ; Fernald et al., 1990]. A comparison of caries experience and Mutans Streptococci and Lactobacilli counts between CF homozygotes, heterozygotes and controls, revealed a significantly lower caries experience (DMF-S) in CF homozygotes than in CF heterozygotes, but no significant difference between CF homozygotes and controls [Aps et al., 2001].

The aim of the present study was to evaluate cross sectionally the caries experience (DMF) and oral cleanliness between CF homozygotes, CF
heterozygotes and healthy controls in Belgium, and to investigate potential correlations.

5.2. Material and methods

5.2.1. Patient selection

CF homozygotes (n = 42) were recruited from the Belgian Association for the Fight Against cystic Fibrosis (BAFCF). Their mean age was 16.2 ± 8.1 years old. Genetically proven CF heterozygotes (n = 48) were also recruited via BAFCF. They were related (as parents or mostly older brother or sister) to the selected CF homozygotes in this study. Consequently their mean age was 29.5 ± 15.9 years old. Healthy controls (n = 62), excluding subjects suffering from cardiovascular, genito-urinary, endocrine, hematological or infectious diseases, were recruited from the Ghent University Dental out-patient clinic and from the CF patients’ and CF heterozygotes’ environment. CF patient family members were excluded as control subject. Their mean age was 19.9 ± 11.5 years old.

Prior to participation, all participants were informed about the aims of the study and all signed an informed consent. The study was approved by the Ghent University’s medical ethical committee.

5.2.2. Caries experience

The investigator (J.A.) was trained at baseline and participated both in an in vitro and in vivo calibration exercise, of which details and results were published elsewhere [Martens et al., 2001]. Caries experience was recorded according to the WHO [http://www.whocollab.od.mah.se] and the British Association for the Study of Community Dentistry (BASCD) guidelines [Pine et al., 1997; Pitts et al., 1997]. Caries was recorded at the level of cavitation and no radiographs were taken. All subjects were examined by the same investigator in a horizontal position and examinations were carried out at the Ghent University dental out-patient clinic or at
the subject’s home. The latter was necessary as not all participants were able to visit the clinic or because, in case of CF homozygotes, they were appropriately afraid to get infected by *Pseudomonas aeruginosa*, which gives a worse prognosis for CF patients. All data were immediately stored in an automated database on a Compaq® Armada 1700 computer.

5.2.3. Dental plaque and calculus

As the purpose of this study was to obtain data on the presence and not on the amount of dental plaque and calculus per tooth or per surface (with exception of the occlusal surface), and as in most cases subjects were examined at home, a fast and easy recording technique was considered most appropriate. In order to compare data from literature, all teeth, except the third molars were involved. Visual presence of plaque and calculus were scored "1" and absence was scored as "0". In case of doubt, a CPI probe [http://www.whocollab.od.mah.se] was used for verification. Consequently the presence of dental plaque and calculus was recorded as a numerical value, both on tooth and on surface level.

5.2.4. Bleeding index

The gingival status was recorded after assessing caries experience, dental calculus and dental plaque. A simplified version of the Löe gingival index (GI) [Löe, 1967] was used; score "0" was absence of bleeding after gentle probing with a CPI probe [http://www.whocollab.od.mah.se] and scores 1 to 3 were converted into one score (score "1") corresponding to bleeding after gentle probing. This was recorded for every tooth present (apart from the third molars) and for the respective surfaces. For the reasons mentioned above, as it was not the aim of the study to investigate the severeness of the gingival status, no extensive bleeding index notation was considered necessary.
5.3. Statistics

All data were immediately stored in an Access-database on a Compaq® Armada 1700 computer. Appropriate statistical analysis was performed using the Kruskal-Wallis test to investigate differences for caries experience and oral cleanliness among the three groups, while the Mann-Whitney U-test was used to compare differences between two groups. The non-parametric Spearman rank correlation was used to investigate correlations between the caries experience data and the oral cleanliness data in every group. The level of significance was set at \( \alpha = 0.05 \).

5.4. Results

5.4.1. Caries experience

In table 5.1. caries experience data are shown with the respective composing factors, for both tooth and surface level. This table also illustrates all P-values after statistical analyses.

We observed that the caries experience (DMF-T) was significantly different between the three groups (\( P = 0.001 \)). CF homozygotes had a significantly lower caries experience than both CF heterozygotes (\( P = 0.011 \)) and controls (\( P < 0.001 \)). The difference between CF heterozygotes and controls was borderline not significant (\( P = 0.06 \)).

Present decay was not significantly different between the three groups, while missing teeth due to caries, was (\( P < 0.001 \)). CF heterozygotes had significantly more missing teeth than controls (\( P = 0.008 \)) and the latter had significantly more missing teeth than CF homozygotes (\( P < 0.001 \)).

Considering the three groups, there seemed to be no significant difference for restored teeth, the restoration’s extensiveness, however, was (\( P = 0.046 \)). CF homozygotes had significantly smaller restorations compared to CF heterozygotes (\( P = 0.015 \)), while this was borderline significant compared to healthy controls (\( P = 0.054 \)).
Table 5.1: Caries experience (DMF-T, D-T, M-T, F-T, DMF-S, D-S, M-S, F-S) and oral cleanliness (dental calculus, dental plaque and gingival bleeding both on tooth -T- and on surface -S- level) data with the results of the statistical analyses for CF homozygotes (n = 42), CF heterozygotes (n = 48) and healthy controls (n = 62)

<table>
<thead>
<tr>
<th></th>
<th>CF Homoz. (A) mean (SD)</th>
<th>CF Heteroz. (B) mean (SD)</th>
<th>Controls (C) mean (SD)</th>
<th>Kruskal-Wallis test P-value</th>
<th>Mann-Whitney U-test P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N = 42</td>
<td>4.05 (5.35)</td>
<td>9.94 (8.12)</td>
<td>6.79 (6.11)</td>
<td>0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>N = 48</td>
<td>4.05 (5.35)</td>
<td>9.94 (8.12)</td>
<td>6.79 (6.11)</td>
<td>0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>N = 62</td>
<td>4.05 (5.35)</td>
<td>9.94 (8.12)</td>
<td>6.79 (6.11)</td>
<td>0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>DMF-T</td>
<td>0.31 (0.78)</td>
<td>0.48 (1.09)</td>
<td>0.61 (1.33)</td>
<td>0.562</td>
<td>0.288</td>
</tr>
<tr>
<td>D-T</td>
<td>0.31 (0.78)</td>
<td>0.48 (1.09)</td>
<td>0.61 (1.33)</td>
<td>0.562</td>
<td>0.288</td>
</tr>
<tr>
<td>M-T</td>
<td>0.26 (0.89)</td>
<td>3.62 (6.92)</td>
<td>0.94 (2.13)</td>
<td>&lt;0.001</td>
<td>0.067</td>
</tr>
<tr>
<td>F-T</td>
<td>3.52 (4.63)</td>
<td>5.88 (6.01)</td>
<td>5.34 (5.17)</td>
<td>0.100</td>
<td>0.043</td>
</tr>
<tr>
<td>DMF-S</td>
<td>9.43 (16.17)</td>
<td>34.94 (36.24)</td>
<td>19.97 (23.05)</td>
<td>&lt;0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>D-S</td>
<td>0.50 (1.25)</td>
<td>0.94 (2.28)</td>
<td>1.40 (4.11)</td>
<td>0.561</td>
<td>0.287</td>
</tr>
<tr>
<td>M-S</td>
<td>1.31 (4.43)</td>
<td>18.44 (34.61)</td>
<td>4.68 (10.67)</td>
<td>&lt;0.001</td>
<td>0.067</td>
</tr>
<tr>
<td>F-S</td>
<td>7.62 (12.55)</td>
<td>15.56 (18.48)</td>
<td>13.89 (16.75)</td>
<td>0.046</td>
<td>0.015</td>
</tr>
<tr>
<td>Plaque-T</td>
<td>3.62 (5.67)</td>
<td>2.63 (4.44)</td>
<td>2.85 (3.87)</td>
<td>0.514</td>
<td>0.258</td>
</tr>
<tr>
<td>Calculus-T</td>
<td>1.93 (3.05)</td>
<td>2.77 (3.82)</td>
<td>2.10 (3.46)</td>
<td>0.423</td>
<td>0.244</td>
</tr>
<tr>
<td>Gingival</td>
<td>0.24 (0.79)</td>
<td>2.35 (5.15)</td>
<td>1.15 (2.38)</td>
<td><strong>0.033</strong></td>
<td><strong>0.014</strong></td>
</tr>
<tr>
<td>bleeding-T</td>
<td>0.24 (0.79)</td>
<td>2.35 (5.15)</td>
<td>1.15 (2.38)</td>
<td><strong>0.033</strong></td>
<td><strong>0.014</strong></td>
</tr>
<tr>
<td>Plaque-S</td>
<td>5.02 (9.68)</td>
<td>5.06 (11.08)</td>
<td>5.11 (8.73)</td>
<td>0.649</td>
<td>0.378</td>
</tr>
<tr>
<td>Calculus-S</td>
<td>3.10 (4.86)</td>
<td>5.17 (7.62)</td>
<td>3.82 (7.46)</td>
<td>0.343</td>
<td>0.202</td>
</tr>
<tr>
<td>Gingival</td>
<td>0.24 (0.80)</td>
<td>5.33 (13.15)</td>
<td>1.93 (5.09)</td>
<td><strong>0.026</strong></td>
<td><strong>0.017</strong></td>
</tr>
<tr>
<td>bleeding-S</td>
<td>0.24 (0.80)</td>
<td>5.33 (13.15)</td>
<td>1.93 (5.09)</td>
<td><strong>0.026</strong></td>
<td><strong>0.017</strong></td>
</tr>
</tbody>
</table>

Note: Significant values are highlighted in bold.
5.4.2. Oral cleanliness

No significant differences were observed between the three groups or between any two groups, for the number of teeth or surfaces covered with dental plaque or dental calculus (table 5.1.). On the other hand, the number of bleeding sites was significantly different between the three groups, both on tooth (P = 0.033) and on surface level (P = 0.026). A Mann-Whitney U-test revealed that CF homozygotes had significant less gingival bleeding sites than CF heterozygotes (P = 0.014 on tooth level and P = 0.011 on surface level) and controls (P = 0.019 on tooth level and P = 0.017 on surface level).

5.4.3. Correlations

No correlations in any group were found between caries experience and oral cleanliness.

Table 5.2. summarizes the correlation values between the oral cleanliness parameters on tooth level and their respective P-values. No correlations were found in CF homozygotes between any of the oral cleanliness parameters. In CF heterozygotes, however, positive significant correlations were found between dental calculus and plaque (r = 0.414 and P = 0.003) and between dental calculus and gingival bleeding (r = 0.470 and P = 0.001). In healthy controls, positive significant correlations were observed between gingival bleeding and dental calculus (r = 0.472 and P < 0.001) and between gingival bleeding and dental plaque (r = 0.316 and P = 0.012). Although the significancies were very pronounced (P ≤ 0.012), the correlations were rather weak (r < 0.5).

5.5. Discussion

It should be emphasized that this study clearly made a difference between CF heterozygotes and control subjects. Nevertheless, statistically the control group could potentially contain 2 more CF heterozygotes. In the past [Kinirons, 1983;
Kinirons, 1985; Kinirons, 1989] this differentiation was not possible, as at that
time the responsible chromosome and gene (7q31) was not yet determined. It was
interesting, however, to notice that CF heterozygotes, who are unaffected by this
disease and as such could be considered as “healthy” controls, had experienced
significantly more extractions than the control group, and that they had
significantly more and extensive restorations than CF homozygotes. This can partly

Table 5.2. : Non-parametric Spearman rank correlations and their statistical
significance between the oral cleanliness parameters on tooth level
(dental calculus, dental plaque and gingival bleeding) for CF
homozygotes (n = 42), CF heterozygotes (n = 48) and healthy controls
(n = 62)

<table>
<thead>
<tr>
<th>Correlations</th>
<th>Dental calculus versus Dental plaque</th>
<th>Dental calculus versus Gingival bleeding</th>
<th>Dental plaque versus Gingival bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CF homozygotes</td>
<td>r = 0.167</td>
<td>r = 0.118</td>
<td>r = 0.289</td>
</tr>
<tr>
<td>N = 42</td>
<td>P = 0.290</td>
<td>P = 0.458</td>
<td>P = 0.063</td>
</tr>
<tr>
<td>CF heterozygotes</td>
<td>r = 0.414</td>
<td>r = 0.470</td>
<td>r = 0.159</td>
</tr>
<tr>
<td>N = 48</td>
<td>P = 0.003</td>
<td>P = 0.001</td>
<td>P = 0.282</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>r = 0.128</td>
<td>r = 0.472</td>
<td>r = 0.316</td>
</tr>
<tr>
<td>N = 62</td>
<td>P = 0.322</td>
<td>P &lt; 0.001</td>
<td>P = 0.012</td>
</tr>
</tbody>
</table>

be explained by the fact that the mean age of the CF heterozygote group
individuals was substantially higher than in both other groups. The CF
heterozygote group consisted of parents, brothers and sisters of the CF
homozygotes, which automatically explains a higher mean age.

This study (figure 5.1.) confirmed that CF homozygotes had a significant lower
caries experience than control subjects [Kinirons, 1985; Kinirons, 1989],
moreover, it became obvious that they also had a significant lower caries experience than CF heterozygotes. This group was never investigated in the past, except in a recent study in which bacterial counts were also involved [Aps et al., 2001]. In the latter study, however, no significant differences were found between CF heterozygotes and CF homozygotes, which probably can be explained, in part, by the small sample used.

The earlier reported higher dental calculus incidence in CF homozygotes [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990] was not confirmed in this study. Moreover, no significant differences between the three groups were found for this parameter, nor for plaque. This is also illustrated in figure 5.1.

On the other hand, CF homozygotes showed significantly less gingival bleeding than both other groups, while no significant differences were observed between CF heterozygotes and controls (figure 5.1.). Both findings, a significantly lower caries experience and significantly less gingival bleeding sites, could be related to the lower, although not significant, sites with plaque, which probably contained a low pathogenic microflora. Alternatively, certain dietary components, such as dairy products, are more prominently present in a CF homozygote’s diet which is mostly sugar rich [Bentur et al., 1996]. Despite this caries risk behaviour, CF homozygotes showed a significantly lower caries experience. Moreover, certain intrinsic salivary processes could render them more protected against dental decay too. It is also suggested in the literature that CF homozygotes have a significantly higher salivary buffer capacity and a higher salivary calcium concentration [Kinirons, 1983]. Both the latter and other salivary electrolytes could also contribute to this low caries profile.
Figure 5.1: Illustration and brief summary of caries experience (DMF-T) and oral cleanliness (plaque, calculus and gingival bleeding) for CF homozygotes (n=42), CF heterozygotes (n=48) and healthy controls (n=62).

It should not be forgotten that CF homozygotes consume a lot of antibiotics, which could potentially reduce the oral bacterial cariogenic flora, resulting in a lower caries experience [Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990]. Apparently this may also result in a lower plaque pathogenicity and may be explain their improved gingival health, as suggested in the present study.

Pointing to one particular aspect of oral hygiene behaviour or dietary habits as the responsible agent for dental decay, plaque, calculus and gingival bleeding...
would be inappropriate. The weak, but nevertheless very significant correlations, support the concept that oral cleanliness (dental plaque, calculus and gingival bleeding) and caries experience are multifactorial conditions.

5.6. Conclusion

From this study it is clear that CF heterozygotes can not be considered as another group of "healthy" individuals as they apparently differ from healthy controls in caries experience. Despite the fact that CF homozygotes potentially run a high caries risk, they do not develop more carious lesions than healthy individuals. CF homozygotes had significantly less gingival bleeding than controls and CF heterozygotes. The latter could involve dietary and oral hygiene aspects, intrinsic salivary processes and/or medication use as agents responsible for lessening carious lesions or pathogenic plaque formation, both of which should be subject for further research.
Chapter 6

Mutans streptococci, Lactobacilli and caries experience in cystic fibrosis homozygotes, heterozygotes and healthy controls

This chapter has been published as:
ABSTRACT

*Mutans Streptococci (MS)* and *Lactobacillus (LB)* counts (CRT® Bacteria chair side test) were compared and correlated with the respective caries experience (DMF) of cystic fibrosis (CF) homozygotes (n = 20), CF heterozygotes (n = 20) and healthy controls (n = 20). Each group of subjects was divided into two equal age groups; A<20y and B>20y. No significant differences were found regarding DMF between the three groups. CF heterozygotes had a significant higher DMF-S than CF homozygotes (P=0.040). This was also found in age group B (P<0.02), where it also counted for DMF-T (P<0.01). When in age group B, CF heterozygotes and controls were combined as one group, it was found that they had a significant higher DMF-T (P=0.031) and DMF-S (P=0.008) than CF homozygotes. No significant differences in LB counts between the three groups were observed. CF homozygotes and those in age group A, had significant (P=0.018 and P = 0.015) more low MS counts. LB and MS counts were positive and significantly correlated with DMF-T/-S in CF homozygotes and in the combined group of CF heterozygotes with controls (P ≤ 0.05 and P ≤ 0.001). No correlations between bacterial counts and DMF were observed in CF heterozygotes. It can be concluded that despite the fact that CF homozygotes behave as high caries risk individuals, they did not have significant more dental decay. More research is required in order to explore salivary, dietary and medication influences.
6.1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians, with a prevalence of 1 in every 2000 live-births. About 4% of the white Belgian population is heterozygous for the cystic fibrosis transmembrane regulator (CFTR) gene [Wauters et al, 1991]. The disease is characterized by an increased mucoid secretion by all exocrine glands, among which are the salivary glands [Hughes and Griffith, 1984; Bellon and Gilly, 1990; Tsui, 1991; Aitkin and Fail, 1993; Hodson, 1993; Sheppard and Ostegaard, 1996; Hilman, 1997]. Salivary calcium and chlorine are altered under these circumstances [Jiménez-Reyes and Sanchez-Aguirre, 1996]. But it is, however, not known if this phenomenon in combination with an increased salivary viscosity has any effect on oral microflora and consequently on caries experience. For a long time it was suggested that young CF patients have significant lower caries experience than controls [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990]. The latter was, however, not confirmed in a recent age matched-control study [Martens et al., 2001] with a variety of young and older CF patients.

The present study aimed to evaluate mutual differences in Mutans Streptococci (MS) and Lactobacilli (LB) counts between CF homozygotes, CF heterozygotes and healthy controls. In a second part, these data were correlated with caries experience.

6.2. Material and methods

6.2.1. Patient selection

CF homozygotes (n = 20) and CF heterozygotes (n = 20) were both recruited from the Belgian Association for the Fight against Cystic Fibrosis. All CF heterozygotes were related to the CF homozygotes and genetically proven to be heterozygous for the CFTR gene, which avoided expensive and unethical genetic
identification. Healthy controls (n = 20), excluding subjects suffering from cardiovascular, endocrine, hematological, infectious or genito-urinary diseases, were recruited from the Ghent University’s dental out-patient clinic. All three groups were subsequently divided into two age groups; age group A < 20 years old and age group B > 20 years old (twice n = 10). For CF homozygotes, ages varied between 9 and 34, for CF heterozygotes between 7 and 60 and for controls between 8 and 39. The study was approved by the University’s ethical committee and all participants signed an informed consent.

6.2.2. Saliva sampling method and CRT®
CRT® Bacteria (Vivadent, Liechtenstein) was chosen as a commercial bacterial colony counting test kit, as it is simple to perform and provides the opportunity to evaluate both MS and LB on the same two-sided dip-slide [Rogosa et al., 1951; Gold et al., 1973; Larmas, 1975; Kneist et al., 1999,a,b]. The CRT® Bacteria test-results are expressed as a low (< 10^5 CFU) or a high (≥ 10^5 CFU) bacterial count. Chair-side tests, such as CRT® Bacteria and Dentocult® (Orion Diagnostica, Finland) have been extensively described in literature as being reliable and easy to use clinically to assess caries risk in populations [Twetman et al., 1994; Kneist and Heinrich-Weltzien, 1997; Kneist et al., 1998; Kneist et al., 1999,a,b; Twetman et al., 1999].

Paraffin stimulated whole saliva was collected in a sterile plastic receptacle. Samples were collected between 9 a.m. and 4 p.m. in the presence of the same investigator (J.A.). Dipslides were inoculated, incubated and evaluated according to the manufacturer’s guidelines. All data (including the caries experience data) were immediately stored in an automated database (Excel file on a Compaq® Armada 1700) for further statistical analysis.
6.2.3. Investigator calibration, CRT® evaluation and Caries experience data collection

Prior to the study, the investigator (J.A.) was trained in evaluating the CRT® Bacteria commercial test results. Subsequently the investigator evaluated a total of 40 samples twice with an interval of 8 hours (to avoid major changes on the agar plates) to establish intra-examiner reliability, which was determined as kappa = 0.71. The investigator was also trained [Martens et al., 2001] to evaluate caries experience (DMF-T/-S) [Klein et al., 1938] according to the British Association for the study of Community Dentistry (BASCD) guidelines [Pine et al., 1997; Pitts et al., 1997]. Only a white light source, cotton rolls, a dental mirror and a CPI probe were used to examine the subjects [WHO, 1997]. Caries was detected at the level of cavitation without the use of X-rays.

6.3. Statistics

The results of the CRT® Bacteria tests were dichotomized (< $10^5$ CFU versus $\geq 10^5$ CFU). The Pearson Chi-Square test was performed to evaluate differences in MS and LB counts between the three groups, while differences in caries experience between the 3 groups were investigated by means of the Kruskal-Wallis test. A Mann-Whitney-U test was used to investigate numerical data between two (age) groups mutually. The non-parametric Spearman rank correlation was calculated between the DMF-index and CRT® counts in the 3 groups. The level of significance was set at $\alpha = 0.05$.

6.4. Results

6.4.1. DMF data

The caries experience data, expressed as DMF-T/-S (mean, standard deviation and median), for all groups and their respective age groups, are summarized in
table 6.1. The respective P-values are also included in this table. No significant differences were observed between the three groups for DMF-T/-S. Comparing them in pairs, it was found that CF homozygotes had a significant lower DMF-S than CF heterozygotes (P = 0.040). Irrespective of the group, age group A individuals had significantly lower DMF-T/-S values than individuals from age group B. The respective p-values ranged between P < 0.001 and P = 0.011 (not included in table 6.1.). When only age group B individuals (> 20 y olds) were evaluated, it was observed that DMF-S was significantly lower (P = 0.022) in CF homozygotes than in CF heterozygotes and controls of age group B. Comparing the respective pairs, it was observed that this difference was only significant between CF homozygotes and CF heterozygotes and between CF homozygotes and all other subjects in age group B (P < 0.01). The latter was also true for DMF-T (P = 0.031).

6.4.2. CRT® results

Table 6.2. comprizes the number of low and high MS and LB counts for every group and age group. No significant differences were found in LB counts nor between the 3 groups nor when the age groups were evaluated. Conversely, it was found that significantly more « low MS counts » were recorded in CF homozygotes (P = 0.018) than in CF heterozygotes or controls. When CF heterozygotes and controls were considered as one group, this difference was even more pronounced (P = 0.009).

CF homozygotes in age group A showed significant more « low MS counts » (P= 0.015) than their pairs of CF heterozygotes or controls. This difference was even more pronounced when CF heterozygotes and controls in age group A were considered as one group (P = 0.007).
Table 6.1: Mean, standard deviation and median caries experience data for CF homozygotes (n = 20), CF heterozygotes (n = 20), healthy controls (n=20) and their respective agegroups (agegroup A< 20y old and agegroup B> 20y old). Results of Kruskal-Wallis test and Mann-Whitney-U test are also included. Significancies are typed in bold.

<table>
<thead>
<tr>
<th></th>
<th>CF Homozygotes “A”</th>
<th>CF heterozygotes “B”</th>
<th>Controls “C”</th>
<th>Kruskal-Wallis test</th>
<th>Mann-Whitney-U-test</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A⇒B⇒C</td>
<td>A⇒B</td>
</tr>
<tr>
<td><strong>DMF-T</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>4.4 (6.0)</td>
<td>8.6 (7.6)</td>
<td>6.3 (7.2)</td>
<td>P = 0.212</td>
<td>P = 0.081</td>
</tr>
<tr>
<td>Agegroup A</td>
<td>1.3 (2.0)</td>
<td>2.2 (2.9)</td>
<td>1.3 (2.2)</td>
<td>P = 0.646</td>
<td>P &gt; 0.10</td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>0.5</td>
<td>1.0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agegroup B</td>
<td>7.4 (8.0)</td>
<td>15.0 (4.9)</td>
<td>10.8 (7.3)</td>
<td>P = 0.063</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>5.5</td>
<td>16.0</td>
<td>7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DMF-S</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>10.0 (18.0)</td>
<td>27.9 (28.4)</td>
<td>20.1 (28.0)</td>
<td>P = 0.126</td>
<td>P = 0.040</td>
</tr>
<tr>
<td>Agegroup A</td>
<td>1.9 (3.5)</td>
<td>4.8 (8.5)</td>
<td>3.0 (4.7)</td>
<td>P = 0.627</td>
<td>P &gt; 0.10</td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>0.5</td>
<td>1.5</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agegroup B</td>
<td>18.0 (22.9)</td>
<td>51.0 (21.1)</td>
<td>35.4 (31.5)</td>
<td>P = 0.022</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Mean (SD) median</td>
<td>9.5</td>
<td>49.0</td>
<td>17.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.2: Distribution of high (≥ 10⁵ CFU) and low (< 10⁵ CFU) bacterial counts for CF homozygotes (n = 20), CF heterozygotes (n = 20), control subjects (n = 20) and the respective agegroups (n = 10) (agegroup A < 20y old and agegroup B >20y old)

<table>
<thead>
<tr>
<th></th>
<th>Lactobacillus Low counts</th>
<th>Lactobacillus High counts</th>
<th>Str. Mutans Low counts</th>
<th>Str. Mutans High counts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CF Homozygotes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 20)</td>
<td>11</td>
<td>9</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Agegroup A (n=10)</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><strong>CF Heterozygotes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=20)</td>
<td>7</td>
<td>13</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Agegroups A (n=10)</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Agegroups B (n=10)</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td><strong>Control subjects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=20)</td>
<td>7</td>
<td>13</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Controls subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agegroup A (n=10)</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Control subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agegroup B (n=10)</td>
<td>3</td>
<td>7</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

6.4.3. CRT® correlations

Positive significant correlations between MS count and LB count were only found in control subjects (r = 0.55, P = 0.013) and in the combined group of CF heterozygotes plus controls (r = 0.41, P = 0.008).
6.4.4. DMF and CRT® Correlations

Table 6.3. shows the correlations, with their respective P-values, between CRT® counts and DMF data. The significant correlations between the caries experience data and the CRT® Bacteria counts were all positive.

In CF homozygotes, MS counts and LB counts were both significantly correlated with DMF-T/-S. CF heterozygotes, showed no significant correlations, whatsoever, between caries experience and bacterial counts. Healthy controls, showed a significant correlation between MS counts and DMF-T/-S. In the combined group of CF heterozygotes with controls, both MS and LB counts showed a significant correlation with DMF-T/-S.

Table 6.3. : Correlations with their respective significancies (* P \leq 0.05 and ** P \leq 0.001) between caries experience (DMF-T and DMF-S) and bacterial counts (**Lactobacillus –LB– and Mutans Streptococci –MS–) for CF homozygotes (n=20), CF heterozygotes (n=20), healthy controls (n=20) and the combined group of CF heterozygotes and controls (n=40)

<table>
<thead>
<tr>
<th>Group</th>
<th>Caries Experience</th>
<th>SM counts</th>
<th>LB counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF homozygotes</td>
<td>DMF-T</td>
<td>0.47*</td>
<td>0.69**</td>
</tr>
<tr>
<td></td>
<td>DMF-S</td>
<td>0.49*</td>
<td>0.68**</td>
</tr>
<tr>
<td>CF heterozygotes</td>
<td>DMF-T</td>
<td>0.31</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>DMF-S</td>
<td>0.24</td>
<td>0.18</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>DMF-T</td>
<td>0.52*</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>DMF-S</td>
<td>0.52*</td>
<td>0.39</td>
</tr>
<tr>
<td>CF heterozygotes +</td>
<td>DMF-T</td>
<td>0.36*</td>
<td>0.31*</td>
</tr>
<tr>
<td>healthy controls</td>
<td>DMF-S</td>
<td>0.33*</td>
<td>0.34*</td>
</tr>
</tbody>
</table>
6.5. Discussion

Knowing that about 4 to 5% of the Caucasian population is heterozygous for the CFTR gene [Wauters et al., 1991], implies that, in previous studies [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Martens et al., 2001], among control subjects, CF heterozygotes were always present. As a consequence the present study is different from others, as it differentiates between CF homozygotes, CF heterozygotes and “healthy controls”. It should be stressed, however, that the authors were well aware that therefore the “healthy control group” potentially and statistically contained at least 1 CF heterozygote.

In general, CF patients did not have a significant lower caries experience than controls, which was in agreement with a recent age-matched case control study on DMF in CF patients [Martens et al., 2001] and in disagreement with earlier reports [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989]. The present analysis however, showed in contrast to the Martens study, that in age group B, CF homozygotes had a significant lower DMF-S than CF heterozygotes and controls. They also had a significant lower DMF-T and –S than the combined group of CF heterozygotes with controls in this age group. This should be interpreted with care, as each age group B only consisted of 10 individuals. CF heterozygotes did have a significant higher caries experience at surface level (DMF-S) compared to CF homozygotes, but not compared to controls.

This study indicated that CF homozygotes did not have a significant different caries experience than control subjects and CF heterozygotes together, irrespective of the fact that CF homozygotes, out of sheer necessity, consume many in-between-meal-sugar-rich-snacks and that they are “chronic users” of aerosols, which are both considered as high risk factors for developing caries [Martens and Robberecht, 1995; Kankaala et al., 1998; Kargül et al., 1998].

There was only a significant difference in DMF-S between CF homozygotes and CF heterozygotes, from which can be concluded that CF homozygotes had less
extensive decay than CF heterozygotes. The latter is an unexpected, but interesting fact as it is in contrast with an earlier finding [Kankaala et al., 1998; Kargül et al., 1998] on asthmatic patients, another respiratory disease, in which aerosols are also used on a regular basis. Under the above mentioned potential “ideal” circumstances, MS should flourish, but the opposite was found. Nevertheless, the significant lower MS count did not result in a significant lower caries experience for CF homozygotes in general.

It was postulated [Martens et al., 2001] that CF patients might have “compensatory salivary mechanisms”, which protect them from extensive dental decay, despite their potential high risk behaviour. The present study puts this statement into perspective, as certain dietary components, such as milk and sweets, and on the other hand the daily use of antibiotics probably play an important role in the development of caries in CF homozygotes. The latter clearly needs further investigation as one should, however, take into consideration that antibiotics may prevail over the use of frequent in-between-meals and drinks.

It is remarkable that no significant correlations between caries experience and CRT®, whatsoever, were observed in CF heterozygotes and no significant differences between CF heterozygotes and control subjects for DMF or CRT® results were observed either. As clinically, CF heterozygotes are not distinguishable from the general population, they are not affected by the CFTR mutation, and could therefore be considered as one group with controls versus CF homozygotes in the statistical analyses.

6.6. Conclusion

Despite the fact that CF homozygotes could be expected to run a potential higher risk for dental decay, no significant differences were observed in caries experience between CF homozygotes and healthy controls. CF heterozygotes even had a significant higher DMF-S than CF homozygotes. CF homozygotes had
significantly more low $MS$ counts than both other groups. Apparently CF homozygotes seem to be protected against high $MS$ colonization by « salivary compensatory mechanisms » or dietary and medication influences, which apparently do not result in a significant lower caries experience, compared to healthy controls with low $MS$ counts. A larger, multicentre study is recommended, to evaluate caries experience differences between these 3 groups. As dental decay is a multifactorial infectious disease, it is obvious that many factors may be of particular interest for further research in assessing differences in salivary composition (organic and inorganic) and salivary (intrinsic and extrinsic) biochemical processes in CF homozygotes.
Chapter 7

The influence of medication and dietary habits on caries experience and oral cleanliness in cystic fibrosis homozygotes

This chapter was submitted for publication as:


Part of this chapter was rewarded with the Société Française d’Odontologie Pédiafrique-Vivadent award 2001
ABSTRACT

Cystic fibrosis (CF) homozygotes (n = 42), heterozygotes (n = 48) and healthy controls (n = 62) were examined to obtain data on caries experience (DMF) and oral cleanliness (dental calculus, dental plaque and gingival bleeding), oral hygiene habits and medication use (vitamins, aerosols, antibiotics and pancreas enzymes). Results: CF homozygotes had significantly less caries experience and gingival bleeding sites than both other groups, while CF heterozygotes had experienced significantly larger dental decay. Vitamin preparations, aerosols and antibiotics did not affect caries experience or oral cleanliness in any of the three study groups. CF homozygotes who opened their pancreas enzyme capsules did experience less dental calculus than those who did not. CF homozygotes consumed significantly more dairy products than both other groups, which can partly explain their lower caries experience. It was concluded that the dairy substances in a CF patient’s diet played a significant role in the caries experience and gingival health of CF homozygotes, while the role of antibiotics in general was not significant.
7.1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians. About 4 to 5% of the Belgian Caucasian population is heterozygous for the CFTR (cystic fibrosis transmembrane regulator) gene, which is located on the long arm of chromosome 7 (7q31). Although several hundreds of genetic mutations are known, the most common mutation is Delta-F508. The prevalence of the disease is 1 in every 2000 live newborns. In essence, the disease affects all exocrine glands (including the salivary glands) and renders their respective secretions more viscous than under healthy conditions. CF patients consequently suffer from gastro-intestinal (due to pancreatic insufficiency) and respiratory problems. Due to this, they are compelled to have supplemental pancreas enzymes with every meal, drink and snack, if necessary supplemental vitamins (A, D, E and K), frequent antibiotics (per os and I.V.) and daily expectorantia and mucolytica (mostly via aerosols) [Hughes and Griffith, 1984; Tsui, 1991; Webb, 1991; Aitken, 1993; Hodson, 1993; Sheppard and Ostegaard, 1996; Hilman, 1997].

Literature on CF and oral health is rather scarce. Study results published in the 1980’s and the beginning of the 1990’s, claimed that CF youngsters (mean age 7.01 years old) had significantly less caries experience than control subjects [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990]. This was attributed to their higher salivary buffer capacity, higher salivary calcium concentration and their frequent use of antibiotics. The latter findings were not confirmed in a recent, age matched control study, performed on a CF group with a wider age range (mean age 16.3 years old). There seemed to be no significant difference in caries experience between CF homozygotes and healthy controls [Martens et al., 2001].

More recently, CF homozygotes were compared with CF heterozygotes and healthy controls. From this study it became clear that CF homozygotes had
significantly less salivary *Mutans streptococci* colonies, despite their many in-between-meals and drinks. Further it was shown that CF homozygotes had a significant lower caries experience (DMF-T) than both CF heterozygotes and controls and that they experienced significantly less gingival bleeding than both other study groups [Aps et al., 2001; Aps et al., 2002a].

The frequent use of antibiotics was reported to be responsible for a lower caries experience in CF patients. It was postulated, although not scientifically proven, that *Mutans streptococci* would decrease in number when antibiotics are taken on a frequent basis [Kinirons, 1985]. On the other hand several studies in medicine have shown a tremendous increase in bacterial resistance against several currently used antibiotics and that secondary infections may be the unfortunate result [Appelbaum, 1992; Levy, 1998; Jones, 1999; Magee et al., 1999; Marsh and Martin, 1999c; Rubinstein, 1999; Ameyes, 2000; Virk and Steckelberg, 2000]. Therefore it can be expected that also in the oral cavity a shift in microflora is possible in subjects taking antibiotics on a frequent basis for years on end. The latter could explain why in a former study less *Mutans streptococci* were detected in CF homozygotes than in heterozygotes and healthy controls [Aps et al., 2001]. The exact effect of antibiotics on caries experience, however, is not known.

Further, one can also assume, as suggested in recent literature, that CF homozygotes potentially run a high caries risk due to their essential sucrose rich diet with many in-between-meal snacks and drinks [Martens and Robberecht, 1995; Martens et al., 2001; Aps et al., 2001; Aps et al., 2002a] and their frequent use of aerosols, of which is also known that it bears a potential caries risk [Kankaala et al., 1998; Kargül et al., 1998; McDerra et al., 1998].

The present study aimed to investigate the use of medication and the differences in dietary habits in CF homozygotes, heterozygotes and healthy controls, analysed against caries experience, dental plaque, dental calculus and sulcus bleeding as response variables.
7.2. Material and methods.

7.2.1. Patient selection

CF homozygotes (n = 42) were recruited from the Belgian Association for the Fight Against Cystic Fibrosis. Their mean age was 16.2 ± 8.1 years. Via the CF homozygotes, genetically proven CF heterozygotes (n = 48) were contacted. Due to ethical issues, no CFTR genetic identification could be performed on control subjects as permission was not granted by the Ghent University Hospital’s ethical committee. As a consequence CF heterozygotes were mainly older brothers or sisters and parents of the CF homozygotes, which automatically resulted in a mean age of 29.5 ± 15.9 years for this study group. Healthy controls (n = 62), excluding CF homozygote related persons, subjects suffering from cardiovascular, genito-urinary, endocrine or infectious diseases, were recruited from the Ghent University Dental out-patient clinic and from the CF patients’ and CF heterozygotes’ environment. Their mean age was 19.9 ± 11.5 years.

The study was approved by the Ghent University Hospital’s ethical committee and all participants signed an informed consent.

7.2.2. Caries experience and oral cleanliness parameters

Prior to the study, the investigator (J.A.) was trained and calibrated at baseline according to the guidelines from the British Association for the Study of Community Dentistry [Pine et al., 1997; Pitts et al., 1997]. The investigator’s overall kappa values for the in vitro and in vivo calibration exercise were 0.84 and 0.76 respectively, both standing for an acceptable interreliability with an experienced examiner as benchmark [Martens et al., 2001]. All clinical examinations were carried out by the same investigator (J.A.), with the subjects in a horizontal position. A white light source, a WHO/CPITN-type E probe, cotton rolls and a dental mirror were the only tools used. No radiographs were taken. Most of the CF
homozygotes and heterozygotes were examined at home, as many CF homozygotes were appropriately afraid to become infected with *Pseudomonas* in a hospital environment. Caries experience was expressed as DMF-T and DMF-S. Caries was recorded at the level of cavitation. Oral cleanliness parameters, a recording of dental plaque, dental calculus and gingival bleeding, were collected from every participant. As the purpose of this study was to obtain data on the presence and not on the amount of dental plaque and calculus per tooth or per surface (with exception of the occlusal surface), and as in most cases subjects were examined at home, a fast and easy recording technique was considered most appropriate. In order to compare data from literature, all teeth, except the third molars were involved. Visual presence of plaque and calculus were scored "1" and absence was scored as "0". In case of doubt, a CPI probe [WHO, 1997] was used for verification. Consequently the presence of dental plaque and calculus was recorded as a numerical value, both on tooth and on surface level [Aps et al., 2002a].

The gingival status was recorded after assessing caries experience, dental calculus and dental plaque. A simplified version of the Loe gingival index (GI) [Loë, 1967] was used; score "0" was absence of bleeding after gentle probing with a CPI probe [WHO, 1997] and scores 1 to 3 were converted into one score (score "1") corresponding to bleeding after gentle probing. This was recorded for every tooth present (apart from the third molars) and for the respective surfaces. For the reasons mentioned above, as it was not the aim of the study to investigate the severeness of the gingival status, no extensive bleeding index notation was considered necessary [Aps et al., 2002a].

7.2.3. Medication inquiry

Every subject was asked to make a list of all medication they took on a regular basis or which they had been taking for a long period of time (= maintenance rations). The aim of this part of the study was to verify what kind of medication
had any influence on the caries experience and oral cleanliness of the subjects in this study. These data were subsequently catalogued into 3 categories; vitamin preparations, antibiotics, aerosols and others. The category of aerosols could contain mucolytica, expectorantia or antibiotics. The “others” category was not used for further statistical analysis, as it contained irrelevant data, such as anticonceptiva.

In case of CF patients, the number of pancreas enzyme capsules (Pancrease® or Creon®) as well as the mode of intake (open or closed capsules) were recorded. The number of capsules per meal were dichotomized (< 10 capsules / meal and ≥ 10 capsules / meal) as this was considered most appropriate for further statistical analysis.

7.2.4. Dietary habits

The investigator (J.A.) interviewed every subject, to obtain information on their dietary habits. The interview was based on a questionnaire, which was used with success in the past and of which the validity was shown [Rise et al., 1991a; Rise te al., 1991b; Goldbohm et al., 1994; Osler and Heitman, 1996; Vanobbergen, 2001; Bolin et al., 1997ab]. The authors had obtained experience, working with this questionnaire, during their collaboration with the “Children’s dental health in Europe” project [Bolin et al., 1997b]. It was accepted that possible noise would lead to an equal shift of the results, but as statistical analyses models were used with a dichotomised outcome and explanatory variables, this was to be minimised [Vanobbergen, 2001]. As the subjects were aware that the investigator was a dentist, specific questions about cariogenic food substances (e.g. sweets, soft drinks and fruitjuices) were banned from the interview. As a consequence, the investigator could filter biassed answers, which are usually difficult to distinguish in a written questionnaire filled in by the subjects themselves.
The interviewer probed for the composition of every meal and snack as well as the time of the day when was eaten and drunk and how and where this was carried out. During the interview, the investigator immediately recorded all data in an Access® data file (Microsoft® Corporation). Some data were immediately dichotomized during input (e.g. “I have breakfast every day / I do not have breakfast every day”) and others were recorded more into detail (e.g. “I drink tea / coffee / tea with sugar / coffee with sugar / … at breakfast”). In order to facilitate the statistical analyses, answers were listed into categories.

Drinks were divided into 4 categories, being; “no drinks, sweetened drinks (e.g. fruit juices, soft drinks, coffee with sugar), non sweetened drinks (e.g. black coffee, plain tea, water) and dairy drinks (e.g. milk, yogurt, coffee with milk)”.

Foods were divided into 3 categories, being; ”a hot meal, a sandwich-like meal with a sweet filling (e.g. marmalade) and a non-sweet filling (e.g. meat, cheese, vegetables, salads)”. The desserts were categorized into 4 groups, being; “no dessert, a sweet dessert (e.g. pie, candy bar), fruit and a non-sweet dessert (e.g. cheese)”. Snacks were divided into drinks and food, being; “no drink, a sweetened drink (e.g. fruit juices, soft drinks, coffee with sugar), a non-sweetened drink (e.g. plain tea or coffee), dairy drinks (milk, yogurt, tea with milk), a sweet snack (e.g. candy bar, cookies), fruit and non-sweet snacks (e.g. a cheese or ham sandwich)”.

7.3. Statistical analysis

The statistical analysis was performed within specific categories (see Table 7.1.) by means of the SPSS program. Pearson chi-square tests were used to investigate differences in dietary habits between the three groups of subjects. The Mann-Whitney U-test was used to investigate differences between dietary habits and medication use with caries experience and oral cleanliness as response variables. The level of significance was choosen at $\alpha \leq 0.05$. 

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7.4. Results

7.4.1. Caries experience and oral cleanliness

Table 7.1 shows the caries experience and oral cleanliness data for the three study groups. From this table it was clear that CF homozygotes had a significant lower caries experience than CF heterozygotes ($P = 0.011$) and controls ($P < 0.001$). The extensiveness of the caries experience (DMF-S) was also significantly lower in CF homozygotes than in both other groups ($P = 0.004$ and $P < 0.001$ respectively).

Table 7.1: Mean caries experience and oral cleanliness data for CF homozygotes (n = 42), CF heterozygotes (n = 48) and healthy controls (n = 62) and the results of the statistical analyses

<table>
<thead>
<tr>
<th></th>
<th>CF homoz. (A)</th>
<th>CF heteroz. (B)</th>
<th>Controls (C)</th>
<th>Kruskal-Wallis Test</th>
<th>Mann-Whitney U-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 42</td>
<td>N = 48</td>
<td>N = 62</td>
<td>$A\leftrightarrow B\leftrightarrow C$</td>
<td>$A\leftrightarrow B$</td>
</tr>
<tr>
<td>DMF-T</td>
<td>4.05 (5.35)</td>
<td>9.94 (8.12)</td>
<td>6.79 (6.11)</td>
<td>0.001</td>
<td>0.011</td>
</tr>
<tr>
<td>DMF-S</td>
<td>9.43 (16.17)</td>
<td>34.94 (36.24)</td>
<td>19.97 (23.05)</td>
<td>&lt; 0.001</td>
<td>0.004</td>
</tr>
<tr>
<td>Surfaces with plaque</td>
<td>5.02 (9.68)</td>
<td>5.06 (11.08)</td>
<td>5.11 (8.73)</td>
<td>0.649</td>
<td>0.378</td>
</tr>
<tr>
<td>Surfaces with calculus</td>
<td>3.10 (4.86)</td>
<td>5.17 (7.62)</td>
<td>3.82 (7.46)</td>
<td>0.343</td>
<td>0.202</td>
</tr>
<tr>
<td>Surfaces with gingival bleeding</td>
<td>0.24 (0.80)</td>
<td>5.33 (13.15)</td>
<td>1.93 (5.09)</td>
<td><strong>0.026</strong></td>
<td><strong>0.011</strong></td>
</tr>
</tbody>
</table>
7.4.2. Medication effects

Aerosols, vitamin preparations or antibiotics did not have a significant influence on caries experience or oral cleanliness in any of the three groups. Figure 7.1. shows the results for the CF homozygotes. It should be stressed that obviously, very few CF heterozygotes and control subjects used this kind of medications on a regular basis.

**Figure 7.1.** Mean caries experience (DMF-T and DMF-S) and oral cleanliness data (mean number of teeth with plaque, calculus and gingival bleeding) as a function of the type of medication in cystic fibrosis homozygotes (n = 42)
Sixty nine percent of CF homozygotes were under aerosol maintenance ration, 57% of them under vitamin preparation maintenance ration and 26% under antibiotics maintenance ration. The pancreas enzymes, which were obviously only recorded to be taken daily in the CF homozygote group, appeared to play a role in the occurrence of dental calculus (fig. 7.2.). CF patients (18%) who opened their enzyme capsules had significantly less teeth with dental calculus than those who did not (P = 0.033).

7.4.3. Dietary habits

From table 7.2., it can be concluded that significantly (P = 0.050) more CF homozygotes than CF heterozygotes had dairy drinks for breakfast. It was further shown that more CF homozygotes than controls had a drink with their forenoon snack. About 50% of control subjects had no drink whatsoever.

The type of drink, during the forenoon snack, was also significantly different (P = 0.001) between the three groups. CF homozygotes had more dairy drinks and sweetened drinks (e.g. Coca Cola®) and less non-sweetened drinks than the others. The latter was confirmed (P < 0.001) when CF homozygotes were compared with controls.

There was a significant difference in lunch composition between the three groups (P = 0.042). CF homozygotes had significantly (P = 0.035) more often a hot meal for lunch than controls. The latter also held for CF heterozygotes (P = 0.035), when compared to control subjects. CF homozygotes also had significantly (P = 0.023) more often a sweetened dessert after lunch than controls.

The composition of the afternoon drinks was significantly different (P = 0.020) between the three groups. Significantly more (P = 0.013) CF homozygotes than controls subjects had a sweetened or dairy drink with their afternoon snack. Significantly fewer (P = 0.017) CF homozygotes had fruits as a snack in the afternoon than controls.
Significantly ($P = 0.044$) more CF homozygotes had sweet fillings for diner than the others, while most controls seemed to have a hot meal for diner. Significantly more ($P = 0.014$) CF homozygotes than CF heterozygotes had a dairy drink during diner.

Significantly more ($P = 0.019$) CF homozygotes than controls had a late-night snack, which was in most cases non-sweetened ($P = 0.021$), in combination with ($P = 0.016$) dairy or sweetened drink.

**Figure 7.2.** Mean caries experience (DMF-T and DMF-S) and oral cleanliness data (mean number of teeth with plaque, calculus and gingival bleeding) as a function of the mode of pancreas enzyme intake in cystic fibrosis homozygotes ($n = 42$)
Table 7.2: Descriptive data and statistical comparison of the dietary habits of CF homozygotes (-A-), CF heterozygotes (-B-) and healthy controls (-C-) (-Pearson Chi-square test - significant differences are typed in bold)

<table>
<thead>
<tr>
<th></th>
<th>-A-</th>
<th>-B-</th>
<th>-C-</th>
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<td>N = 62</td>
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<td>P</td>
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<td>P</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>No drink with breakfast</td>
<td>86%</td>
<td>90%</td>
<td>90%</td>
<td>0.749</td>
<td>0.405</td>
<td>0.337</td>
<td>0.571</td>
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<td>10%</td>
<td>10%</td>
<td>0.132</td>
<td>0.050</td>
<td>0.744</td>
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<td>21%</td>
<td>48%</td>
<td>27%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy drink with breakfast</td>
<td>38%</td>
<td>19%</td>
<td>32%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Warm breakfast</td>
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<td>23%</td>
<td>23%</td>
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<td></td>
</tr>
<tr>
<td>Sweet breakfast</td>
<td>71%</td>
<td>56%</td>
<td>61%</td>
<td>0.581</td>
<td>0.245</td>
<td>0.508</td>
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<td>21%</td>
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<tr>
<td>Forenoon snack or drink (yes)</td>
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<td>75%</td>
<td>68%</td>
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<td>0.442</td>
<td>0.430</td>
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<td>31%</td>
<td>52%</td>
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<tr>
<td>Sweetened drink with the snack</td>
<td>43%</td>
<td>27%</td>
<td>15%</td>
<td>0.001</td>
<td>0.066</td>
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<tr>
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<td>21%</td>
<td>13%</td>
<td>7%</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Sweet snack</td>
<td>31%</td>
<td>21%</td>
<td>16%</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fruit for snack</td>
<td>2%</td>
<td>10%</td>
<td>11%</td>
<td>0.481</td>
<td>0.376</td>
<td>0.146</td>
<td>0.922</td>
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<td>21%</td>
<td>24%</td>
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<tr>
<td>Lunch (yes)</td>
<td>93%</td>
<td>98%</td>
<td>98%</td>
<td>0.256</td>
<td>0.259</td>
<td>0.179</td>
<td>0.685</td>
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<td>8%</td>
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</tr>
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<td>48%</td>
<td>38%</td>
<td>40%</td>
<td>0.256</td>
<td>0.058</td>
<td>0.471</td>
<td>0.308</td>
</tr>
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<td>54%</td>
<td>45%</td>
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</tr>
<tr>
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<td>7%</td>
<td></td>
<td></td>
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</tr>
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<td>48%</td>
<td>26%</td>
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<td>16%</td>
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<td>0.363</td>
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<td>82%</td>
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<td>0.247</td>
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<tr>
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<td>0%</td>
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<tr>
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<td>83%</td>
<td>71%</td>
<td>69%</td>
<td>0.244</td>
<td>0.125</td>
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<td>38%</td>
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<tr>
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<td>38%</td>
<td>19%</td>
<td>29%</td>
<td><strong>0.020</strong></td>
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<td>35%</td>
<td>24%</td>
<td></td>
<td></td>
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<td>17%</td>
<td>8%</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet snack</td>
<td>29%</td>
<td>25%</td>
<td>15%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fruit for snack</td>
<td>5%</td>
<td>10%</td>
<td>18%</td>
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<td>23%</td>
<td>19%</td>
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<td></td>
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<tr>
<td><strong>Diner (yes)</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>100%</td>
<td>100%</td>
<td>0.268</td>
<td>0.467</td>
<td>0.404</td>
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<td>21%</td>
<td>29%</td>
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<td>58%</td>
<td>45%</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hot meal for diner</td>
<td>14%</td>
<td>0%</td>
<td>8%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Sweet fillings with diner</td>
<td>67%</td>
<td>65%</td>
<td>81%</td>
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<td>0.157</td>
<td>0.053</td>
<td>0.165</td>
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<tr>
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<td>29%</td>
<td>16%</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>No dessert after diner</td>
<td>55%</td>
<td>63%</td>
<td>60%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet dessert after diner</td>
<td>41%</td>
<td>33%</td>
<td>32%</td>
<td>0.846</td>
<td>0.594</td>
<td>0.710</td>
<td>0.750</td>
</tr>
<tr>
<td>Fruits after diner</td>
<td>5%</td>
<td>2%</td>
<td>7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-sweet desserts after diner</td>
<td>0%</td>
<td>2%</td>
<td>2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Late-night snack or drink (yes)</strong></td>
<td>83%</td>
<td>71%</td>
<td>63%</td>
<td>0.079</td>
<td>0.125</td>
<td><strong>0.019</strong></td>
<td>0.252</td>
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<td>33%</td>
<td>42%</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>36%</td>
<td>21%</td>
<td>29%</td>
<td><strong>0.016</strong></td>
<td><strong>0.012</strong></td>
<td>0.073</td>
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<td>40%</td>
<td>23%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy drink with late-night snack</td>
<td>21%</td>
<td>6%</td>
<td>7%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet late-night snack</td>
<td>14%</td>
<td>6%</td>
<td>8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit for late-night snack</td>
<td>5%</td>
<td>8%</td>
<td>8%</td>
<td>0.083</td>
<td>0.064</td>
<td><strong>0.021</strong></td>
<td>0.937</td>
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<tr>
<td>Non-sweet late-night snack</td>
<td>57%</td>
<td>38%</td>
<td>32%</td>
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</tr>
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</table>
7.4.4. Correlations between dietary habits and caries experience and oral cleanliness

It was found that control subjects had a significant lower caries experience both on tooth \( (P = 0.008) \) as on surface \( (P = 0.019) \) level when they had dairy products for breakfast. They also had significant less dental calculus \( (P = 0.026) \) than controls having no dairy products for breakfast. Control subjects who had sweetened drinks with their lunch had significantly less dental plaque \( (P= 0.033) \) and calculus \( (P= 0.001) \) than those who had another drink.

When their lunch consisted of sweet fillings, it was found that these controls had significantly \( (P = 0.014) \) less dental calculus than those who had something else for lunch.

Control subjects, having a late-night snack, had a significantly \( (P = 0.043) \) higher DMF-S than those who did not have a late-night snack. For DMF-T this difference was borderline not significant \( (P = 0.054) \).

CF heterozygotes who had breakfast, showed significantly more dental calculus \( (P = 0.018) \) than those who did not have breakfast at all. It was observed that those who had a breakfast, which was composed of sweetened substances, had significantly less dental calculus \( (P = 0.044) \) and a significant lower caries experience on tooth \( (P = 0.025) \) and on surface level \( (P = 0.016) \). CF heterozygotes who had a sweetened forenoon drink, had significantly less caries experience, both on tooth \( (P = 0.005) \) and on surface level \( (P = 0.007) \) than those who had something else to drink. The latter finding was confirmed for drinking a sweetened drink during lunch \( (P = 0.017 \) and \( P = 0.018 \) respectively). CF heterozygotes, who had a late-night snack or drink, had significantly more caries experience, both on tooth \( (P = 0.013) \) as on surface level \( (P = 0.031) \), than those who did not eat or drink late at night.
CF homozygotes who had a sweet dessert after diner, had a significant lower caries experience, both on tooth (P = 0.014) as on surface level (P = 0.019), than CF homozygotes who did have a piece of fruit as dessert or no dessert at all.

7.5. Discussion
This study showed that the influence of medication, such as aerosols and antibiotics, on oral cleanliness and caries experience in CF patients was rather moderate and insignificant. The latter is unexpected as in earlier reports on CF patients, it was postulated that the frequent use of antibiotics was partly responsible for a significant lower caries experience in CF homozygotes [Kinirons, 1985]. As the effect of pancreas enzyme capsules was never investigated before, it was surprising that CF homozygotes who opened their pancreas enzyme capsules had significantly less dental calculus than those who did not open them. Although no explanation can be found immediately for this finding, it deserves to be investigated into depth, whether certain constituents of these pancreas enzyme capsules could be potential anti-tartar agents.

Dietary habits were clearly different between the three groups, which, in turn, can be partly held responsible to explain the differences in caries experience. From this study it became clear that sweetened food substances are not necessarily cariogenic. The time of the day and the combination with other food substances seemed to play a significant role. The latter supported recent reports on the cariogeneicity of food substances [Duggal and Van Loveren, 2001; Van Loveren and Duggal, 2001].

CF homozygotes consumed significantly more dairy products than the others. The effects of milk and cheese on caries development have been described and investigated in the past [König, 1967; Edgar et al., 1975; Rugg-Gunn et al., 1978]. In particular calcium, phosphate, casein, IgA and fats were investigated and it was concluded that dairy products could be considered as being anticariogenic.
Therefore, it can be assumed that the more dairy products are consumed, the more frequent the oral environment is buffered, due to phosphate and calcium. As the latter is not the most favorite ecological environment, the number of *Mutans Streptococci* would decrease considerably. This can probably explain the earlier reported findings [Aps et al., 2001], showing that apparently CF homozygotes seemed to be protected against *Mutans Streptococci* attacks, which occur preferably in sucrose rich environments.

Casein, an enzyme, incorporated in cheese and milk [Reynolds et al., 1981; Van Loveren and Van der Weijden, 1996b] could also be the key to the low caries experience in CF homozygotes. If casein indeed enhanced enamel remineralization, it compensated the expected cariogenic effect of sucrose rich supplements, which CF homozygotes are obliged to take at regular moments of the day. If enhancement of enamel remineralization actually plays an important role, an increase of the mineral content (i.e. calcium phosphate) should be present in CF homozygotes. If so, the latter would imply that the above mentioned frequent intake of antibiotics is not the only extrinsic determining factor for their significant reduced caries experience as already suggested in earlier studies [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990].

It was interesting to find that the use of dairy products also resulted in caries experience and oral cleanliness differences in control subjects and CF heterozygotes. The use of dairy products seemed to result in a significant lower amount of dental calculus within both controls and CF heterozygotes, but not within CF homozygotes. The significant lower number of teeth covered with plaque and calculus in controls that consumed a sweetened drink during lunch, showed that sweetened drinks within a meal did not affect oral cleanliness negatively, as might be expected. In CF heterozygotes, breakfast seemed to have great impact on dental calculus and caries experience. It seemed, however, that the
importance of certain kinds of foods and drinks for the development of caries, plaque and calculus, should not be exaggerated too much.

Moreover, it has to be further investigated if the better gingival status in CF homozygotes can be attributed to a better oral health care in this particular group of medically compromised patients or to particular salivary properties which protect them from gingival inflammation.

The fact that caries experience was significantly higher in CF heterozygotes than in CF homozygotes, can, however, not be explained by their dietary habits alone [Duggal and Van Loveren, 2001; Van Loveren and Duggal, 2001]. It has to strike one’s mind that CF heterozygotes in this study were all related to the CF homozygotes.

As such, they all lived in the same environment and had globally the same food, except less dairy products. Nevertheless, they had experienced more dental decay than their CF homozygote family members. Besides the higher mean age of the CF heterozygote group, which obviously was caused by the CF heterozygotes mainly being parents and older brothers and sisters of the CF homozygotes, the aspect of oral hygiene should be taken into account as well. Perhaps, CF heterozygotes are not that aware of their oral hygiene as are CF homozygotes, who are constantly remembered of microorganisms, threatening their live.

Finally it can be assumed that differences in salivary composition are responsible for CF heterozygotes experiencing more dental decay than both CF homozygotes and control subjects.

7.6. Conclusion

It was concluded that dietary habits (increased intake of dairy products) can partly be held responsible for the differences in caries experience and oral cleanliness between CF homozygotes, CF heterozygotes and controls. The anticariogenic effect of dairy products during meals and in-between-meals can be
appointed as one of the compensatory mechanisms in the multicausal caries process. The supposed negative influence of regular use of aerosols or antibiotics on oral health was not confirmed in this study and therefore intrinsic salivary properties in CF homozygotes probably may also be held partly responsible for their significant better oral health.

Acknowledgement
This study was financially supported by the Fund for Scientific Research in Flanders, Belgium (FWO; grant 3-0014-98) and logistically supported by the Belgian Association for the Fight against Cystic Fibrosis.
Chapter 8

Oral hygiene habits and oral health in cystic fibrosis

This chapter was submitted for publication as:

ABSTRACT

Oral hygiene habits in cystic fibrosis (CF) homozygotes (n = 42), heterozygotes (n = 48) and healthy controls (n = 62) were used in a multivariate analysis with caries experience (DMF-T) and oral cleanliness (dental plaque, calculus and gingival bleeding) as response variables.

CF homozygotes had a significantly lower caries experience (P ≤ 0.001) and less gingival bleeding sites (P ≤ 0.02). Oral hygiene habits were not significantly different between the three groups, except for intake of fluoride supplements. Significantly more CF homozygotes had received fluoride supplements. Oral hygiene habits did affect caries experience or oral hygiene differently in each study group.

No matter what oral hygiene habits were, CF homozygotes had an overall better oral health status. Apparently they seemed to possess intrinsic salivary compensatory mechanisms, as the significant higher use of fluoride supplements appeared not to be responsible for the better oral health.
8.1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians. About 4 to 5% of the Caucasian population is heterozygous for the CFTR (cystic fibrosis transmembrane regulator) gene, which is located on the long arm of chromosome 7 (7q31). Although several hundreds of genetic mutations are known, the most common mutation in Western Europe is Delta-F508. The prevalence of the disease is 1 in every 2000 live newborns. In essence, the disease affects all exocrine glands (including the salivary glands) and renders their respective secretions more viscous than under healthy conditions. CF patients consequently suffer from gastro-intestinal (due to pancreatic insufficiency) and respiratory problems. Due to this, they are compelled to have supplemental pancreas enzymes with every meal, drink and snack, supplemental vitamins (A,D,E and K) if necessary, frequent antibiotics (per os, via aerosols and I.V.) and daily expectorantia and mucolytica (mostly via aerosols) [Hughes and Griffith, 1984 ; Tsui, 1991 ; Webb, 1991 ; Aitken, 1993 ; Grundy et al., 1993 ; Hodson, 1993 ; Vay Liang et al., 1993 ; Sheppard and Ostegaard, 1996 ; Hilman, 1997].

Literature published in the 1980’s and the beginning of the 1990’s, claimed that CF youngsters (mean age 7.0 years) had significantly less caries experience than control subjects [Kinirons, 1983 ; Kinirons, 1985 ; Kinirons, 1989 ; Fernald et al., 1990]. This was attributed to their higher salivary buffer capacity, higher salivary calcium concentration and their frequent use of antibiotics. A more recent age matched control study, performed on a CF group with a wider age range (mean age 16.3 years) [Martens et al., 2001], reported no significant differences in caries experience between CF homozygotes and healthy controls. Due to lack of diagnostic tools, no information on CF heterozygotes was available in the 1980’s and as a consequence they were unavoidably included in the healthy control study groups.
Recently a study [Aps et al., 2001] was published in which CF homozygotes, CF heterozygotes and healthy controls were compared. No significant differences in caries experience between these 3 groups, were found. CF homozygotes, nevertheless, had significantly less salivary *Mutans Streptococci* counts. In a continuing study [Aps et al., 2002a] on a larger sample of CF homozygotes, CF heterozygotes and healthy controls it was shown that CF heterozygotes had significantly larger restorations than CF homozygotes, while the latter had also a significantly lower caries experience than healthy controls. CF homozygotes were also reported to have significant less bleeding gingival sites than CF heterozygotes and controls.

Several presumptions, dealing with compensatory salivary mechanisms, inherent to their disease, were mentioned in these reports, which could serve as an explanation for CF homozygotes experiencing less dental decay than others, despite the assumption them running a high caries risk (e.g. frequent in-between-sucrose-rich-snacks and drinks and daily aerosols). From an analysis of their dietary habits [Aps et al., 2002b] it was concluded that CF homozygotes may experience positive benefit from consuming lots of dairy products, whether sweetened or not. But as caries is a multifactorial infectious disease, other aspects of influence should be investigated also.

The present study, therefore, aimed to investigate oral hygiene habits in CF homozygotes, heterozygotes and healthy controls. Consecutively, these variables were used in a multivariate analysis with caries experience (DMF-T) and oral cleanliness (dental plaque, dental calculus and gingival bleeding) as response variables.
8.2. Material and methods

8.2.1. Patient selection

Both CF homozygotes (n = 42, 16.2 ± 8.1 years) and genetically proven CF heterozygotes (n = 48, 29.5 ± 15.9 years) were recruited via the Belgian Association for the Fight Against Cystic Fibrosis. The high mean age of the CF heterozygotes can be explained by the fact that the CF heterozygote group consisted of parents and mainly older brothers and sisters of CF homozygotes. Healthy controls (n = 62, 19.9 ± 11.5 years), excluding subjects suffering from cardiovascular, genito-urinary, neurological, endocrine or infectious diseases, were recruited from the Ghent University Dental out-patient clinic and from the CF patients’ and CF heterozygotes’ environment. The study was approved by the Ghent University’s medical ethical committee and for all subjects an informed consent was obtained.

8.2.2. Caries experience and oral cleanliness

The methodology to record caries experience (DMF-T) and oral cleanliness (dental plaque, dental calculus and gingival bleeding) were already extensively described elsewhere [Martens et al., 2001; Aps et al., 2002a]. All teeth, except for the third molars, were included in the recording of the oral cleanliness parameters. Consequently the mean number of teeth with plaque, calculus and gingival bleeding in every study group was obtained.

8.2.3. Patient questionnaire

To avoid misunderstandings, unanswered questions and biasses, every study subject was questioned by the same investigator (J.A.). By interviewing these subjects, the investigator was able to obtain objective information about their oral hygiene attitudes. All answers were dichotomized as “yes” or “no” immediately while entering the data in the database (Microsoft Access 2000, © Microsoft Corporation).
Answers to the following items on oral hygiene measures were obtained after interviewing every subject:

- I brush less than twice a day / I brush at least twice a day
- I do not brush my teeth in the morning / I brush my teeth in the morning
- I do not brush my teeth in the evening / I brush my teeth in the evening
- I use at least a pea size of toothpaste / I use at least one centimetre of toothpaste
- I do not use a fluoride containing toothpaste / I use a fluoride containing toothpaste
- I use a regular toothbrush / I use an electrical toothbrush
- I never use a fluoride containing mouthrinse / I use a fluoride containing mouthrinse
- I never had fluoride supplements (drops or tablets) / I had fluoride supplements
- I never use a chlorhexidine containing mouthrinse / I use a chlorhexidine containing mouthrinse
- I never use dental floss / I use dental floss
- I never use toothpicks / I use toothpicks

8.3. Statistical analysis

Appropriate statistical analysis was performed by means of the SPSS program. A Kruskal-Wallis test was used to investigate differences in caries experience and oral cleanliness between the three groups of subjects. Subsequently a Mann-Whitney U-test was used to investigate these data between two subject groups at a time. A Pearson Chi-square test was used to investigate differences in oral hygiene habits and oral health knowledge. Regression analysis with the oral hygiene habits as covariables and caries experience, dental plaque, dental calculus and gingival
bleeding as response variables was performed. The level of significance was choosen at $\alpha = 0.05$.

8.4. Results
8.4.1. Caries experience and oral cleanliness

It can be derived from table 8.1. that CF homozygotes had significantly less caries experience than CF heterozygotes ($P < 0.001$) and healthy controls ($P = 0.011$) as well as significantly less gingival bleeding than CF heterozygotes ($P = 0.014$) and healthy controls ($P = 0.019$).

CF heterozygotes had experienced significantly more extractions and had significantly more restored teeth than CF homozygotes ($P < 0.001$ and $P = 0.043$ respectively). They also had experienced more extractions than healthy controls ($P = 0.008$).

Table 8.1.: Caries experience (DMF-T) and oral cleanliness (gingival bleeding, dental plaque and dental calculus on tooth level) in CF homozygotes (N = 42), CF heterozygotes (N= 48) and Healthy controls (N = 62)
8.4.2. Oral hygiene habits

Table 8.2 shows that only the fact of having received any fluoride supplements was significantly different between the three groups (P = 0.037). Significantly more CF homozygotes than CF heterozygotes (P = 0.010) had used fluoride supplements (either drops or tablets).

Table 8.2: Descriptive data on oral hygiene habits in CF homozygotes (N = 42), CF heterozygotes (N = 48) and healthy controls (N = 62) and the appropriate statistical analyses results

<table>
<thead>
<tr>
<th></th>
<th>CF homoz N = 42</th>
<th>CF heteroz N = 48</th>
<th>Controls N = 62</th>
<th>Pearson Chi-Square Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>I brush ≥ 2/day</td>
<td>91%</td>
<td>90%</td>
<td>94%</td>
<td>0.736</td>
</tr>
<tr>
<td>I never brush my teeth</td>
<td>0%</td>
<td>2%</td>
<td>5%</td>
<td>0.306</td>
</tr>
<tr>
<td>I brush in the morning</td>
<td>76%</td>
<td>56%</td>
<td>69%</td>
<td>0.118</td>
</tr>
<tr>
<td>I brush in the evening</td>
<td>71%</td>
<td>79%</td>
<td>87%</td>
<td>0.140</td>
</tr>
<tr>
<td>I use ≤ pea size toothpaste</td>
<td>38%</td>
<td>50</td>
<td>50</td>
<td>0.434</td>
</tr>
<tr>
<td>Fluoride toothpaste</td>
<td>100%</td>
<td>96%</td>
<td>97%</td>
<td>0.436</td>
</tr>
<tr>
<td>Electrical toothbrush</td>
<td>24%</td>
<td>25%</td>
<td>18%</td>
<td>0.610</td>
</tr>
<tr>
<td>Fluoride rinse</td>
<td>0%</td>
<td>6%</td>
<td>5%</td>
<td>0.283</td>
</tr>
<tr>
<td>Fluoride supplements</td>
<td>64%</td>
<td>38%</td>
<td>47%</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>Chlorhexidine rinse</td>
<td>5%</td>
<td>2%</td>
<td>5%</td>
<td>0.725</td>
</tr>
<tr>
<td>I floss</td>
<td>10%</td>
<td>23%</td>
<td>18%</td>
<td>0.239</td>
</tr>
<tr>
<td>I use toothpicks</td>
<td>14%</td>
<td>23%</td>
<td>24%</td>
<td>0.444</td>
</tr>
</tbody>
</table>
8.4.3. Effects of oral hygiene habits on caries experience

CF homozygotes who brushed their teeth in the morning (P = 0.009) as well as those who brushed in the evening (P = 0.016) or those who used toothpicks (P = 0.002) had a significantly higher DMF-T.

Caries experience was significantly higher in CF heterozygotes who did not receive any fluoride supplements (P = 0.028) or who used dental floss (P = 0.002). Healthy controls who used a fluoride containing toothpaste (P = 0.038), or those who never got fluoride supplements (P = 0.050) had a significant higher caries experience. The same was observed in control subjects who used dental floss (P = 0.002) or toothpicks (P = 0.033).

8.4.4. Effects of oral hygiene habits on oral cleanliness

CF homozygotes who used an electrical toothbrush experienced significantly more dental calculus (P = 0.042) than those who used a regular toothbrush.

CF heterozygotes who did not brush in the evening encountered significantly more dental calculus (P = 0.015), as did those who never received any fluoride supplements (P = 0.013) or never used toothpicks (P = 0.002). CF heterozygotes who did not get any fluoride supplements also experienced significantly more plaque (P = 0.047), as did those who used toothpicks (P = 0.036). Besides, using toothpicks also resulted in significantly more gingival bleeding (P = 0.018) in this group.

Healthy control subjects who brushed their teeth in the morning (P = 0.029), or who never received fluoride supplements (P = 0.019) or those who used toothpicks (P = 0.031) experienced significantly more dental calculus than those who did the opposite. Gingival bleeding was significantly higher in those healthy controls who brushed less than twice a day (P = 0.044) or never received any fluoride supplements (P = 0.004).
8.5. Discussion

It was interesting that CF homozygotes, a group of medically compromised individuals who could be supposed to run a high caries risk due to their dietary obligations and medication intake [Martens et al., 2001; Aps et al., 2001; Aps et al., 2002a; Aps et al., 2002b], had a significantly lower caries experience than CF heterozygotes and healthy controls (table 8.1.). It was suggested in recent literature that CF homozygotes may in a way be protected against dental decay and that certain salivary mechanisms may be involved [Martens et al., 2001; Aps et al., 2001; Aps et al., 2002a; Aps et al., 2002b; Aps et al., 2002c].

**Figure 8.1a.** Schematical representation of the effect of NOT brushing in the morning or NOT brushing in the evening or NOT having had fluoride supplements or NOT using dental floss or toothpicks on caries experience (mean DMF-T) in CF homozygotes, CF heterozygotes and healthy controls
On the other hand, it was remarkable to notice that CF heterozygotes, who are not clinically affected by the CFTR gene, apparently ran a higher caries risk than healthy controls and CF homozygotes. They also had experienced significantly more extractions than the others and in a previous report [Aps et al., 2002a] it was also mentioned that the extensiveness of their restorations was significantly larger.

The number of teeth with hypertrophic and/or hyperaemic gingival margins was significantly lower in CF homozygotes than in CF heterozygotes. On the other hand it should be emphasized that the number of teeth covered with plaque was the highest in CF homozygotes, although the difference between the three groups was not significant.

**Figure 8.1b.** Schematical representation of the effect of brushing in the morning or brushing in the evening or having had fluoride supplements or using dental floss or toothpicks on caries experience (mean DMF-T) in CF homozygotes, CF heterozygotes and healthy controls.
other micro-organisms than in CF heterozygotes and control subjects [Aps et al., 2001; Aps et al., 2002], which could explain why they had significantly less bleeding sites than CF heterozygotes and controls. Perhaps this supposed less pathogenic plaque flora could also be responsible for CF homozygotes experiencing significantly less dental decay.

This study showed that very little differences can be found between these three groups regarding their oral hygiene habits. Only the fact of having received fluoride supplements of any kind (droplets or tablets) was significantly different. Sixty four percent of CF homozygotes against 38% CF heterozygotes and 48% healthy controls had received fluoride supplements.

**Figure 8.2a.** Schematical representation of the effect of NOT brushing in the morning or NOT brushing in the evening or NOT having had fluoride supplements or NOT using dental plaque or toothpicks on dental calculus (mean CI) in CF homozygotes, CF heterozygotes and healthy controls.
Probably their pediatricians may have prescribed this “medication” additionally to their already extensive list of obligatory medications, inherent to their medical condition. This could be interpreted as a kind of “overtreatment” or reassurance of the patients’ parents.

The effects of oral hygiene habits on caries experience and oral cleanliness in every study group were rather unexpected, sometimes unlogical and consequently, no clear conclusions could be drawn. It should not be forgotten that oral hygiene measurements are the result of certain oral cleanliness features. It is well possible that certain individuals had started using dental floss quite recently because their dentist had advised them to do so. Other oral hygiene habits, except use of fluoride supplements, may have been “influenced” in the same way. Caries experience provides information about an individual’s past and present experience with dental decay, while an inquiry into oral hygiene attitudes reports only about the present habits. Consequently caries experience and current oral hygiene habits may be divergent.

In a pentagon, of which the surface area is a measure of the influence of brushing habits, fluoride supplements, use of dental floss and toothpicks, it can be visualized to what extend these parameters determine caries experience and oral cleanliness (figures 8.1. to 8.4.). When brushing habits, the use of fluoride supplements, dental floss and toothpicks were considered, whatever the oral hygiene habits of CF homozygotes were, they seemed to be of little influence on caries experience (figure 8.1a. and 8.1b.). The latter illustrated once more the “intrinsic” compensatory mechanisms, mentioned before. The same held for dental calculus (figure 8.2a. and 8.2b.) and gingival bleeding (figure 8.3a. and 8.3b.). CF homozygotes seemed, however, to generate, although not significantly, more plaque than the other subjects (figure 8.4a. and 8.4b.), irrespective of their oral hygiene habits.
Figure 8.2b. Schematical representation of the effect of brushing in the morning or brushing in the evening or having had fluoride supplements or using dental floss or toothpicks on dental calculus (mean CI) in CF homozygotes, CF heterozygotes and healthy controls.

Although the only significant difference in oral hygiene habits between the three groups was the fact of having had fluoride supplements, this did not seem to influence caries experience differences between the three groups (figure 8.5a. and 8.5b.). These quadrangles show that whether CF homozygotes did receive fluoride supplements or not, did not affect their caries experience significantly. The fact that CF homozygotes who received fluoride supplements showed more, although not significantly, teeth with calculus, should be interpreted as coincidental. No correlations between fluorides and dental calculus have been reported before.
Figure 8.3a: Schematical representation of the effect of NOT brushing in the morning or NOT brushing in the evening or NOT having had fluoride supplements or NOT using dental floss or toothpicks on gingival bleeding (mean BI) in CF homozygotes, CF heterozygotes and healthy controls.

These findings reinforce once more the assumption that CF homozygotes may possess, not yet identified, intrinsic salivary compensatory mechanisms, which render them less vulnerable for a bad oral health status, as it did not seem to matter what oral hygiene habits they had. Fluoride supplements did not make the difference either between these three study groups. Nevertheless, it should always be remembered that caries is a multifactorial infectious disease and that many other factors, such as medication, which in CF patients in particular will play an important role, should be included in assessing caries risk in every population of medically compromised patients.
**Figure 8.3b.** Schematical representation of the effect of brushing in the morning or brushing in the evening or having had fluoride supplements or using dental floss or toothpicks on gingival bleeding (mean BI) in CF homozygotes, CF heterozygotes and healthy controls.

**Figure 8.4a.** Schematical representation of the effect of NOT brushing in the morning or NOT brushing in the evening or NOT having had fluoride supplements or NOT using dental floss or toothpicks on dental plaque (mean PI) in CF homozygotes, CF heterozygotes and healthy controls.
**Figure 8.4b.** Schematical representation of the effect of brushing in the morning or brushing in the evening or having had fluoride supplements or using dental floss or toothpicks on dental plaque (mean PI) in CF homozygotes, CF heterozygotes and healthy controls.
**Figure 8.5a.** Effect of « I did NEVER receive fluoride supplements » on caries experience (mean DMF-T), gingival bleeding (mean BI), dental calculus (mean CI) and dental plaque (mean PI) in CF homozygotes, CF heterozygotes and healthy controls.
Figure 8.5b: Effect of « I DID receive fluoride supplements » on caries experience (mean DMF-T), gingival bleeding (mean BI), dental calculus (mean CI) and dental plaque (mean PI) in CF homozygotes, CF heterozygotes and healthy controls.

Acknowledgements

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Chapter 9

Salivary Electrolyte Concentrations Are Compounded with Cystic Fibrosis Transmembrane Regulator Genotypes

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Aps J.K.M., Delanghe J., Martens L.C. Salivary electrolyte concentrations are compounded with cystic fibrosis transmembrane regulator genotypes.

ABSTRACT

It is estimated that about 1 in 24 Belgian Caucasians is a cystic fibrosis (CF) heterozygote. Until now, CF heterozygotes can only be identified by genetic decoding (e.g. Inno-Lipa CF2 test®), as they can not be phenotypically distinguished from the general population. The aim of this study was to evaluate differences in salivary electrolyte concentrations (calcium, bicarbonate, chloride, potassium, sodium and phosphate) and salivary osmolarity between CF homozygotes (n = 41), CF heterozygotes (n = 56) and healthy controls (n = 65). By means of non-parametric tests, differences between the three groups were investigated. Several significant differences between the three study groups and among as well as between the different Cystic Fibrosis Transmembrane Regulator (CFTR) genotypes were observed. Significant differences in salivary electrolyte concentrations between individuals with delta F508 mutation and those without and between CF homozygotes and heterozygotes were observed. Several salivary electrolyte concentrations and salivary osmolarity were significantly higher in CFTR genotype individuals. Differences in salivary electrolyte concentrations may partly explain differences in caries experience (DMF-T and DMF-S) between the three study groups.
9.1. Introduction

Cystic Fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians. The prevalence of CF homozygocity is about 1 in every 2000 live births and about 4 to 5% of Caucasians is heterozygous for the Cystic Fibrosis Transmembrane Regulator (CFTR) gene, which is located on the long arm of chromosome 7 (7q31). The most common mutation is called delta-F508 and it occurs in about 74% of (Belgian) CF patients [Wauters et al., 1991; http://www.genet.sickkids.on.ca/cftr/agreement.htm]. It is a deletion of three nucleotides encoding phenylalanine at position 508 in the amino acid sequence. This single gene mutation causes a defective sodium and chloride transport in every exocrine gland (respiratory and intestinal epithelium, as well as salivary glands), which subsequently results in increased mucous secretions. The expression of the disease is both inter-individually as well as inter-racially different [McColley et al., 1991]. Besides delta-F508, more than 950 other mutations are known to the Cystic Fibrosis Genetic Analysis Consortium. They are less frequent and in many cases specific for one family only or for a specific geographical area [http://www.genet.sickkids.on.ca/cftr/agreement.htm].

As CF is a recessive inherited disease, CF heterozygotes can not be distinguished phenotypically from the general population and clinical signs that might raise suspicion of being a CF heterozygote do not exist. As a consequence, most CF heterozygotes are therefore “incidentally” diagnosed when they become parents of or when they are closely related to a CF homozygote newborn. The only possible way of identifying CF heterozygotes is via genetical procedures. The (pilocarpine -) sweat-test, which is used in case of suspicion of an individual being a CF homozygote, is not useful to check for CFTR heterozygosy and no other available electrolyte test has been explored yet [Augarten et al., 1995; Gilbert et al., 1995; ; Stewart et al., 1995; Veeze, 1995; Rosenstein, 1998; Lotem et al., 2000; Massie et al., 2000a; Massie et al., 2000b].
Saliva, which is the most easy body fluid to collect [Mandel, 1990; Haeckel and Hanecke, 1993], could be used to investigate electrolyte concentration differences between CF homozygotes, heterozygotes and control subjects. Studies published on salivary composition of CF homozygotes versus control subjects did not differentiate between CF heterozygotes and controls [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990; Jiménez-Reyes and Sánchez-Aguirre, 1996].

Knowing that about 5% of the Caucasian population is heterozygous for the CFTR gene, implies that in these studies, CF heterozygotes were accidentally mixed with controls. This is understandable, as most of these studies date from before or around 1985, when the genetical information and techniques were not available.

It would be very convenient and clinically relevant to be able to identify CF heterozygotes by investigating their salivary chemical composition. Besides the low-cost aspect and the ease to collect saliva (a non-invasive method), identification of CF heterozygotes could be performed much faster (in infancy) and more adequate than today. A “suspicious” saliva test result could then be the motive to perform genetic identification of the CFTR gene. Furthermore, saliva can be considered as a “model” fluid for studying the effect of the genetic heterogeneity of CF on the composition of secretions from exocrine glands.

The aim of the present study was to investigate differences in salivary electrolyte concentrations (bicarbonate, calcium, chloride, sodium, potassium, phosphate) and salivary osmolarity between CF homozygotes, CF heterozygotes and healthy control subjects. Secondly, it was investigated if any of these analytes were linked to the CFTR genotypes. Additionally, a clinical oral parameter, the caries experience (DMF-T and DMF-S) of the individuals in this study, was also recorded.
9.2. Material and Methods

9.2.1. Sample selection

The Belgian Association for the Fight against Cystic Fibrosis was explained the aims of this study, and 46 CF homozygotes agreed to participate. Their age varied from 6 to 38 years (17.2 ± 7.8 years). Genetically confirmed CF heterozygotes (n = 69), were enrolled in the study. Their age varied from 3 to 61 years old (31.2 ± 15.1 years). Control subjects (n = 64) were selected from the Ghent University’s dental out-patient clinic. Subjects suffering from cardiovascular, endocrine, hematological, neurological, infectious or genito-urinary diseases, were excluded as control subject. Their age varied between 5 and 51 years old (20.7 ± 11.3 years). While the study as a whole was approved by the Ghent University Hospital’s ethical committee, no permission was granted to perform molecular genetics on the control subjects to avoid CF heterozygotes in this group. Consequently, statistically 4 to 5% CF heterozygotes were unavoidably inclosed in the control group. All subjects involved in the study signed a letter of informed consent.

9.2.2. Saliva collection

Paraffin stimulated whole human saliva was collected in a sterile plastic recipient between 9 a.m. and 4 p.m., while subjects were in an upright position. All subjects were instructed to chew a paraffin tablet, as provided in the Vivadent CRT® Bacteria and Buffer test kit (Schaan, Liechtenstein), for at least 3 minutes under the supervision of the investigator (J.A.). Saliva samples were subsequently transported to the laboratory, where they were analysed within 1 hour after collection.

9.2.3. Salivary electrolytes

Prior to chemical analysis, each sample was vortexed for at least 30 seconds and subsequently 3 milliliter was centrifuged for 10 minutes at 1500 g at room
temperature. Subsequently the supernatant was used for chemical electrolyte analysis (respectively calcium, bicarbonate, phosphate, potassium, sodium and chloride concentrations). Salivary osmolarity was also determined.

Measurement of the total calcium concentration was performed by a colorimetric method and inorganic phosphate was quantified by means of a molybdate complexing reaction method. Both procedures were carried out by means of a testkit provided by Roche® (Mannheim, Germany). The bicarbonate concentration was determined enzymatically using commercial reagents from BioMérieux® (Biomérieux, Marcy-les-Etoiles, France). Potassium, sodium and chloride were automatically measured using indirect potentiometry on the Hitachi 747® analyzer (model 100) (Tokyo, Japan). Osmolarity was measured with the “Advanced cryometric osmometer®” (model 3C2, Advanced Instruments) (Needham Heights, Massachusetts, USA).

9.2.4. Genotypes

Both CF homozygotes and heterozygotes agreed, by a signed consent, to provide the investigators information on their CFTR genotype. Seven CF heterozygotes did not have the necessary information anymore and did not want it to be investigated. The data were obtained by the Inno-Lipa CF2® test (Innogenetics, Ghent, Belgium) [http://www.innogenetics.com], which as a standard procedure investigates 8 CFTR mutations; delta F508, G542X, N1303K, 1717-1G-A, W1282X, G551D, R553X and delta I 507 from genetic material collected by buccal mucosa swabs. These mutations cover about 85% of all CFTR mutations in Western Europe.

In case of equivocal results, another 21 less frequent CFTR mutations were investigated [http://www.innogenetics.com]. In this study, genotypes were divided into two subgroups. One subgroup consisted of individuals with delta F508 genotype and the second subgroup of individuals with any other genotype than
delta F508 or heterozygotes of whom no information was available, but of whom it was certain that the mutation was not delta F508.

9.2.5. Caries experience

Caries experience is widely expressed as “DMF-T and DMF-S” in dental literature. The index was first introduced by Klein and Palmer in 1938 [Klein and Palmer, 1938] and has been used ever since in cross-sectional and longitudinal studies on caries experience within populations. The index exists of three parts; “D” stands for actual dental decay, “M” stands for missing teeth due to extractions for reasons of extensive carious lesions which were considered too large to restore and “F” which stands for filled or restored. The second part of the index indicates whether it concerns the number of teeth (“T”) or the number of surfaces (“S”). All teeth, excluding the third molars are involved in the recording of the index. As a consequence, DMF-T can vary between 0 and 28 and DMF-S can vary from 0 to 140.

The caries experience was recorded according to WHO guidelines [http://www.whocollab.od.mah.se] and the investigator (J.A.) was trained at baseline according to the guidelines published by the British Association for the Study of Community Dentistry in 1997 [Pine et al., 1997; Pitts et al., 1997]. The results of the calibration exercises were already published elsewhere [Martens et al., 2001].

9.3. Statistics

Statistical analysis was performed using the MedCalc® software program (MedCalc, Mariakerke, Belgium). To investigate differences between the three samples (CF homozygotes, CF heterzogotes and healthy controls ), a Kruskal-Wallis test was used. The Mann-Whitney U-test was used to investigate statistical differences between two samples. The level of significance was chosen at \( \alpha \leq 0.05 \).
9.4. Results

The different CFTR genotypes, were obtained after a signed consent from the CF patients and their family.

Sixty five point nine per cent were combinations with \textit{delta F508} and 34.1\% did not have a \textit{delta F508} component. The following CFTR mutation combinations were involved in the CF homozygote group; \textit{delta F508} + \textit{delta F508} (n = 21), \textit{delta F508} + 394 \textit{del TT} (n = 1), \textit{delta F508} + \textit{N1303K} (n = 3), \textit{delta F508} + 3849+10kb \textit{C-T} (n = 1), \textit{delta F508} + \textit{G542X} (n = 1), \textit{delta F508} + 3272-26A→G (n = 1), \textit{delta F508} + \textit{unknown mutation} (n = 1), \textit{G542X} + \textit{G542X} (n = 2) and \textit{delta I507} + \textit{G542X} (n = 1).

Fifty point seven percent of CF heterozygotes in this study had a \textit{delta F508} mutation, 4.4\% had an unknown mutation, 17.4\% were other mutations and 27.5\% did not know and did not want to know what mutation they had. The CF heterozygote group comprised the following (single) CFTR mutations; \textit{delta F508} (n = 35), \textit{2183 AA→G} (n = 1), \textit{3849 10kb C→T} (n = 1), \textit{394 del TT} (n = 4), \textit{G542X} (n = 4), \textit{N1303K} (n = 2), 3 unknown CFTR mutations and 19 heterozygotes had no information on their mutation and did not want it to be investigated.

Table 9.1. summarizes the salivary electrolyte concentrations of CF homozygotes, CF heterozygotes and healthy control subjects. Calcium and bicarbonate were excluded as these electrolytes were never significantly different between any of the three subject groups.

Salivary potassium (p = 0.005), sodium (p = 0.004) and phosphate (p = 0.0001) concentrations and salivary osmolarity (p = 0.033) showed to be significantly different between CF homozygotes, CF heterozygotes and controls. When, subsequently CF homozygotes and CF heterozygotes were compared, it was found that potassium (p = 0.002) and phosphate and (p < 0.0001) concentrations were both significantly higher in CF homozygotes. But when CF homozygotes were evaluated against healthy controls, salivary potassium (p = 0.046), sodium
(p = 0.002) and phosphate (p = 0.002) concentrations as well as salivary osmolarity (p = 0.019) were shown to be significantly higher in CF homozygotes.

Table 9.1: Descriptive data (mean ± standard deviation, median and interquartile range) of salivary electrolytes for CF homozygotes (n = 41), CF heterozygotes (n = 56) and healthy controls (n = 65) and the respective statistical analyses results (* p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 and N.S. = not significant)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>CF Homoz n = 41</th>
<th>CF heteroz n = 56</th>
<th>Healthy controls n = 65</th>
<th>Kruskal-Wallis test p-values</th>
<th>Mann-Whitney U-test p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>A ↔ B ↔ C</td>
<td>A ↔ B</td>
</tr>
<tr>
<td>Chloride (mmol / l)</td>
<td>20.3 (6.0)</td>
<td>19.0</td>
<td>18.8 (7.1)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>13.0 – 34.0</td>
<td>12.9 – 44.4</td>
<td>10.1 – 41.0</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Potassium (mmol / l)</td>
<td>24.5 (4.6)</td>
<td>21.7 (2.7)</td>
<td>22.7 (3.6)</td>
<td>*</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>25.5</td>
<td>21.2</td>
<td>22.4</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>16.8 – 34.1</td>
<td>17.5 – 29.5</td>
<td>16.7 – 30.6</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Sodium (mmol / l)</td>
<td>14.5 (7.5)</td>
<td>16.8 (12.2)</td>
<td>11.8 (9.2)</td>
<td>**</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>13.0</td>
<td>8.0</td>
<td>**</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>6.5 – 35.2</td>
<td>3.8 – 42.1</td>
<td>3.0 – 35.6</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Phosphate (mg / l)</td>
<td>153 (34)</td>
<td>125 (27)</td>
<td>134 (30)</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>145</td>
<td>122</td>
<td>130</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>93 – 237</td>
<td>81 – 187</td>
<td>90 – 204</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Osmolarity (Osmo / kgH₂O)</td>
<td>83 (17)</td>
<td>86 (24)</td>
<td>74 (17)</td>
<td>*</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>79</td>
<td>71</td>
<td>**</td>
<td>**</td>
</tr>
</tbody>
</table>
Only salivary sodium (p = 0.012) concentration and salivary osmolarity (p = 0.034) were significantly higher in CF heterozygotes than in healthy controls. The same two analytes were also significantly higher in all subjects with a CFTR mutation than in healthy controls (p = 0.0009 and p = 0.009 respectively).

Table 9.2: Descriptive data (mean ± standard deviation, median and interquartile range) of both CF homozygotes and heterozygotes with (n = 30 and n = 35 respectively) and without (n = 11 and n = 21 respectively) genotype delta F508 and the respective statistical analyses (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and N.S. = not significant)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Δ F508 CF heteroz N = 35</th>
<th>Not ΔF508 CF heteroz N = 21</th>
<th>ΔF508 CF homozyz N = 30</th>
<th>Not Δ F508 CF homozyz N = 11</th>
<th>Mann-Whitney U-Test p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloride (mmol/l)</td>
<td>23.7 (10.3)</td>
<td>8.9 (5.0)</td>
<td>20.3 (6.3)</td>
<td>20.2 (5.5)</td>
<td>* N.S. N.S. *</td>
</tr>
<tr>
<td></td>
<td>22.0</td>
<td>15.0</td>
<td>18.5</td>
<td>19.0</td>
<td>14.0–30.0</td>
</tr>
<tr>
<td></td>
<td>12.4–46.5</td>
<td>13.0–36.9</td>
<td>13.0–34.5</td>
<td>16.8–35.4</td>
<td></td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>21.2 (2.4)</td>
<td>22.6 (8.9)</td>
<td>24.6 (4.3)</td>
<td>24.3 (5.6)</td>
<td>N.S. N.S. ** N.S.</td>
</tr>
<tr>
<td></td>
<td>20.8</td>
<td>22.2</td>
<td>25.5</td>
<td>21.5</td>
<td>16.8–35.4</td>
</tr>
<tr>
<td></td>
<td>17.7–28.2</td>
<td>16.8–29.4</td>
<td>17.1–32.6</td>
<td>16.8–35.4</td>
<td></td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>19.9 (12.6)</td>
<td>11.5 (9.5)</td>
<td>14.6 (7.8)</td>
<td>14.3 (6.8)</td>
<td>** N.S. N.S. N.S.</td>
</tr>
<tr>
<td></td>
<td>19.0</td>
<td>8.0</td>
<td>12.0</td>
<td>12.0</td>
<td>6.0–29.0</td>
</tr>
<tr>
<td></td>
<td>3.5–47.9</td>
<td>4.0–37.8</td>
<td>8.0–38.3</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Phosphate (mg/l)</td>
<td>126 (30)</td>
<td>124 (24)</td>
<td>149 (35)</td>
<td>163 (27)</td>
<td>N.S. N.S. ** ***</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>125</td>
<td>143</td>
<td>163</td>
<td>116–201</td>
</tr>
<tr>
<td></td>
<td>82–188</td>
<td>81–166</td>
<td>92–246</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>Osmolarity (Osmo/kgH₂O)</td>
<td>90 (25)</td>
<td>79 (22)</td>
<td>82 (16)</td>
<td>85 (20)</td>
<td>N.S. N.S. N.S. N.S.</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>72</td>
<td>77</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>58–146</td>
<td>55–122</td>
<td>66–121</td>
<td>66–118</td>
<td>**</td>
</tr>
</tbody>
</table>

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Comparing CF homozygotes with CF heterozygotes and healthy controls together, revealed a significant higher salivary potassium ($p = 0.005$), sodium ($p = 0.048$) and phosphate ($p < 0.0001$) concentration in CF homozygotes.

In Table 9.2, comparisons between the two genotype subgroups are shown. Comparing the two genotypes (delta F508 and non-delta F508) within the CF heterozygotes (D versus E), showed a significantly higher salivary chloride ($p = 0.018$) and sodium ($p = 0.003$) concentration in the delta F508 genotype subgroup. Within the CF homozygote group (F versus G), no significant differences in salivary electrolyte concentrations or salivary osmolarity, whatsoever, were found between the two genotype subgroups.

Comparisons within the delta F508 genotype individuals (D versus F), revealed a significant higher salivary potassium ($p = 0.001$) and phosphate ($p = 0.003$) concentration in CF homozygotes. Within the non-delta F508 CF homozygotes and heterozygotes (E versus G) significant higher salivary chloride ($p = 0.045$) and phosphate ($p = 0.0009$) concentrations were observed in CF homozygotes.

Caries experience (Table 9.3.) at surface level (DMF-S) was significantly lower in CF homozygotes than in healthy controls ($p = 0.025$). This significance was also shown when CF heterozygotes and control subjects were considered as one study group ($p = 0.03$).

### 9.5. Discussion

Saliva is the most accessible body fluid, next to urine, which can be collected without invasive techniques [Mandel, 1990; Haeckel and Hanecke, 1993]. Therefore, it can be collected from everyone, from infants to elderly, healthy or medically compromised, without being a psychological threat to the patient. In this study paraffin stimulated saliva was used in order to obtain whole human saliva and realistic electrolyte concentrations, such as they appear during mastication.
Table 9.3: Descriptive data of caries experience (mean ± SD) for CF homozygotes (n = 49), CF heterozygotes (n = 69) and healthy controls (n = 65) and the statistical analyses results (* P ≤ 0.05 and N.S. = not significant)

<table>
<thead>
<tr>
<th>Analytes</th>
<th>CF Homozygote A</th>
<th>CF Heterozygote B</th>
<th>Healthy Controls C</th>
<th>B + C</th>
<th>Mann-Whitney U-test P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 46</td>
<td>N = 69</td>
<td>N = 65</td>
<td>N = 134</td>
<td>A vs. B</td>
</tr>
<tr>
<td>DMF-T</td>
<td>4.3 (5.7)</td>
<td>7.2 (8.2)</td>
<td>6.3 (6.4)</td>
<td>6.7 (7.4)</td>
<td>N.S.</td>
</tr>
<tr>
<td>DMF-S</td>
<td>10.2 (17.5)</td>
<td>23.6 (33.1)</td>
<td>18.0 (23.9)</td>
<td>20.9 (29.0)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

In contradiction with earlier reports [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990; Jiménez-Reyes and Sánchez-Aguirre, 1996] on salivary electrolytes in CF, in this study no significant differences in bicarbonate or calcium concentrations were observed between CF homozygotes and healthy controls. The latter could be attributed to different techniques used to measure salivary electrolyte concentrations, to circadian rhythm of salivary secretion (different time of the day at which saliva was collected) or to the stimuli used to stimulate salivary secretion.

In the present study, salivary electrolytes of CF heterozygotes were intentionally investigated for the first time. The latter was not possible in the past, as no differentiation between the genotypes and no identification of CF heterozygotes was possible at that time. Nine different homozygote CFTR mutations were involved in this study. As in Western Europe delta F508 is the most common CFTR mutation, obviously delta F508 homozygotes made up for the largest part of this study group. Six known and three unknown or “unidentified” CFTR mutations in CF heterozygotes were involved in this study group. This study is the first ever
to differentiate between CF heterozygotes and controls. Nevertheless, the authors are well aware of the fact that among the control subjects, statistically 4 to 5% CF heterozygotes may be included [Wauters et al., 1991]. On the other hand, this potential 4 to 5% accidental inclusions, did not seem to be bias, as several significant differences in salivary electrolytes were found between CF heterozygotes and healthy control subjects.

Salivary electrolyte concentration differences indeed exist between CF homozygotes and CF heterozygotes. Apparently they are linked to the respective genotypes. When either comparing them irrespective of their genotype or comparing \emph{delta F508} genotype individuals only, potassium and phosphate were always significantly higher in the CF homozygote group. Salivary phosphate was also considerable significantly higher in CF homozygotes when the not-delta F508 individuals were compared. This significant higher salivary phosphate concentration in CF homozygotes may play an important role in their susceptibility for developing dental decay at a lower rate than other individuals. Phosphate in particular, plays an important role in de- and remineralisation of dental enamel [Driessens and Verbeeck, 1990].

Within the CF homozygote group, no significant differences in salivary electrolyte concentrations or salivary osmolarity were found between the two subgroups of CFTR mutations. Within the CF heterozygote group, however, there were significant differences in salivary electrolyte concentrations. The latter could indicate that phenotypical varieties of CF heterozygotes do exist. Until now, only CF homozygotes were considered to have phenotypical varieties within their genotypes.

The fact that CF heterozygotes were reported to have a tendency of an apparently increased caries risk [Aps et al., 2001; Martens et al., 2001; Aps et al., 2002a; Aps et al., 2002b], could consequently be related to their specific salivary electrolyte concentrations. Although, this effect should not be exaggerated, as caries is a
multifactorial infectious disease, which means that besides host factors, oral microorganisms, dietary habits, oral hygiene habits and environmental factors play an important role as well in the development of dental decay.

Clinically, CF homozygotes show increased mucous salivary secretions [Kinirons, 1983; Kinirons, 1985; Kinirons, 1989; Fernald et al., 1990; Jiménez-Reyes and Sánchez-Aguirre, 1996]. Salivary viscosity can be attributed to either a decreased water content of saliva or to an increased concentration of organic and inorganic components. Besides a significant higher total salivary protein concentration [Aps et al., 1999], inorganic salivary inclusions may also influence salivary viscosity in CF homozygotes. The significant higher salivary osmolarity of CF homozygotes with respect to control subjects, illustrated the clinical observation that saliva of CF homozygotes was more viscous. However, no significant difference between CF homozygotes and CF heterozygotes (irrespective of the genotypes) concerning salivary osmolarity was observed. Moreover, also CF heterozygotes had a significant higher salivary osmolarity than healthy controls.

The observed differences in salivary ion composition between the different phenotypes might reflect differences in the composition of other body fluids (e.g. sweat and tears), since CF affects all exocrine glands. Consequently, further studies will be necessary to confirm the present findings in other body fluids. Future research will decide on whether saliva can be used as a diagnostic tool in investigating CF and other exocrine disorders.

Acknowledgements

This study was financially supported by the Fund for Scientific Research, Flanders (FWO grant : 3.00014.98 ) and logistically by the Belgian Association for the Fight against Cystic Fibrosis. The authors especially wish to thank Professor Dr. Ronald M.H. Verbeeck from the Department of Dental Material Science of the University of Ghent for his expertise and advice.
Chapter 10

SDS-PAGE of salivary proteins in cystic fibrosis;
preliminary results

Part of this chapter was presented as:


Awarded with the first scientific prize of the Belgian Academy of Paediatric Dentistry, 2000
ABSTRACT

Paraffin stimulated whole saliva samples of 36 Cystic Fibrosis (CF) homozygotes, 59 CF heterozygotes and 45 healthy control subjects were collected for protein examination purposes. The total protein concentration was determined and subsequently, every sample was examined by coomassie Brilliant R250 stained sodium-dodecyl-sulphate-poly-acryl-amide gel electrophoresis (SDS-PAGE), to investigate differences in salivary protein composition between the three subject groups.

It was found that CF homozygotes had a significant higher salivary protein concentration than CF heterozygotes and controls (P=0.008). The total salivary protein concentration showed to be a diagnostic aid to distinguish CF homozygotes from non-CF homozygotes. At a cut-off concentration of at least 0.86g/l, a sensitivity of 56.3% and a specificity of 89.6% was obtained. By means of linear regression analysis, three distinct salivary protein bands were recognized on the SDS-PAGE gradient (4-15%) gels. Their molecular weight was respectively 242 kDa, 161.5 kDa and 68 kDa. The single band corresponding with 68 kDa was significantly more present in CF homozygote samples (P = 0.036). However, when this band appeared as a triplet, it was significantly more seen in CF heterozygote samples (P = 0.038). In a multiple regression model, with salivary protein concentration and the single band appearance of the 68 kDa protein on SDS-PAGE gel as independent variables and CF homozygotes as response variable, this model showed a predictibility of 39.5% and a multiple correlation coefficient of 0.63 to differentiate CF homozygotes from CF heterozygotes and healthy control subjects.

It was concluded that salivary proteins can be used to differentiate CF homozygotes and CF heterozygotes, but that identification of the distinctive proteins should be subject of further research. The relationship between these salivary properties and oral health should be investigated further into detail.
10.1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive inherited disease and as a consequence CF heterozygotes can not be detected phenotypically in the general population. Thus, most CF heterozygotes are “incidentally” identified when they become parents of or when they are closely related to a CF homozygote newborn. Salivary studies about CF homozygotes and heterozygotes are scarce, as most literature on this issue focussed on bacterial aggregation in saliva and its repercussions on the CF patient’s general health. Especially the role of saliva and aggregation with *Pseudomonas* was investigated. The latter is logical, as CF patients who get infected with *Pseudomonas* will eventually die from this irreversible infection [Tumber-Saini et al., 1992; Armstrong et al., 1993].

It is reported in the literature that the total salivary protein concentration is higher in CF homozygotes than in healthy control subjects [Van Nieuw Amerongen, 1988a,b], but nothing is known about the situation in CF heterozygotes. The latter can be understood as in the past CF heterzygotes could not be identified, while today this is possible by means of genetic decoding.

Therefore, the aims of this study were to evaluate differences in total salivary protein concentrations between CF homozygotes, CF heterozygotes and healthy controls. Secondly, it was investigated whether any distinct differences in salivary composition could be distinguished between these three groups.

10.2. Material and methods

10.2.1. Subject selection

CF homozygotes (n = 36) aged between 6 and 38 years old (16.5 ± 8.0 years), 56 CF heterozygotes aged between 3 and 61 years old (31.4 ± 15.5 years) were recruited via the Belgian Association for the Fight Against Cystic Fibrosis. As the latter were mostly parents and older family members of CF homozygotes, their mean age was obviously higher than those of the CF homozygotes. A total of 45
individuals, aged between 5 and 40 years (19.6 ± 10.5 years), were recruited from the Ghent University Dental Hospital out-patient clinic. Subjects suffering from cardiovascular, endocrine, hematological, infectious or genito-urinary diseases were excluded as control subject.

As obviously no permission was granted by the ethical committee to perform genetic verification of the control subjects, unavoidably 4 to 5% of these individuals were potential CF heterozygotes.

All subjects signed a letter of informed consent and the study was approved by the Ghent University Hospital’s ethical committee.

10.2.2. Saliva collection

A paraffin stimulated saliva sample was collected from every subject. Saliva was collected in a sterile plastic recipient, subjects were in an upright position and the collections were made between 9 AM. and 4 PM, in the presence of the investigator (J.A.). Subsequently the samples were transported to the laboratory, where they were frozen in aliquots of 3 ml, at −17°C and stored for later investigation.

10.2.3. Laboratory procedures

10.2.3.1. Total protein concentration

Two milliliter saliva of a well vortexed sample was centrifuged at 1500g for 10 minutes. Subsequently the supernatant was pipet and the total protein content was determined automatically on a Hitachi® 911 automatic analyzer (Tokyo, Japan), which uses the pyrogallol-red molybdate complexing method [Van Ingen, 1990]. The result is expressed as gram total protein per liter saliva.
10.2.3.2. Electrophoresis

Electrophoresis, in general, is the movement of particles in an electrical field. It is one of the most efficient analytical tools in modern clinical biochemistry to separate the different monomers of a polymer. Saliva can be considered as a polymer and as a consequence, the individual monomers, the salivary proteins, can be identified by means of sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) based on their molecular mass. This technique was preferred over others, which are based on the principle of electrical charge of proteins [Frankel et al., 1970; Henry et al., 1974; Schultze and Heremans, 1966; Beeley, 1991; Beeley et al., 1991; Beeley et al., 1996].

The gel can be considered being a sieve. The gels used in this study were 4-15% gradient gels, which means that at the top of the gel, large proteins (with a high molecular mass) are captured in the sieve, while near the bottom of the gel, the smaller proteins (with a low molecular mass) are retained. This results in distinct banding of proteins, which can be visualised by means of staining with several agents, such as Coomassie Brilliant Blue R250 or silver.

10.2.3.3. SDS-PAGE procedure

Three milliliter saliva sample was ultracentrifuged (Beckman® L-2 centrifuge) at 13,000 rpm for 30 minutes at 10°C. The supernatant was then pipeted and frozen at −17°C. Subsequently it was lyophilized at -70°C (Virtis® Gardiner, NY 12525, USA) for at least 8 hours, until only powder remained. Thereupon, the lyophilized saliva was diluted in 250 microlitre sample buffer (\(pH = 6.8\); 3.8 ml distilled water, 1.0 ml 0.5 \(M\) Tris-HCl, 1.6 ml 10% SDS, 0.8 ml glycerol, 0.4 ml 1% brominephenol blue (Merck®)) and well vortexed for at least 10 seconds. The sample colors immediately blue-purple. In order to separate the sialic acid groups from the salivary glycoproteins, 10 microlitre of neuraminidase was added to an aliquot of 50 microlitre of this solution and incubated for 2 hours at 37°C.
Subsequently the electrophoresis buffer solution (pH = 8.3; 9 g of Tris alkaline + 43.2 g of glycine + SDS + diluted with distilled water to a volume of 600 ml) was prepared.

The SDS- PAGE 4-15% Tris-HCl-gradient gels (BIO-RAD® Laboratories, 2000 Alfred Nobel Drive, Hercules CA 94547, USA), were mounted in the respective Mini-Protean II® electrophoresis cell (BIO-RAD®) and by means of a syringe, 10 microlitre of salivary sample was injected into the indentations of the gel.

SDS-PAGE was performed at a constant voltage of 200 Volts for 50 minutes. The progression of the electrophoresis was easy to follow because of the samples’ blue color.

After 50 minutes, the gels were unmounted from the electrophoresis chamber and moved into a Petri dish and saturated with a Coomassie Brilliant Blue R250 solution (LKB Bromma®, a 1g/l dilution in 40% methanol and 10% acetic acid) for 30 minutes. Subsequently the gels were destained several times in a solution with 40% methanol and 10% acetic acid until only distinct protein bands were visible as blue and purple horizontal lines.

10.2.4. Determination of the molecular masses

By means of a standardized protein mix (SDS-PAGE Standards, broad 200 µl, order number 161-0317), as provided by Bio Rad®, the molecular masses can be derived, by means of linear regression analysis. The reference proteins (from high to low molecular mass) used in this study were myosine, bèta-galactosidase, fosforylase, serum albumine, oval albumine, carbonic anhydrase, trypsin inhibitor and lysozyme.

The gels were scored by two investigators (J.A. and KVDM) and immediately stored in an automated Excel file (Microsoft® Corporation).
10.3. Statistical analysis

Statistical analysis was performed using of the MedCalc® statistical program (MedCalc® Software, Broekstraat 52, B-9030 Mariakerke, Belgium, info@medcalc.be). Linear regression analysis was used to determine the molecular mass of the salivary protein bands on the SDS-PAGE gels. A one-way analysis of variance test was used to investigate differences in total salivary protein concentration between the three study groups. A Kruskal-Wallis test was used to investigate differences between the three groups, with regard to the appearance of the three protein bands on the SDS-PAGE gels. Multiple regression models were investigated with CF homozygotes as response variable and salivary protein concentration and protein bands as independent variables.

10.5. Results

10.5.1. Total salivary protein concentration

It was found that CF homozygotes had a significant higher total salivary protein concentration than CF heterozygotes and healthy control subjects (P=0.008). The mean total salivary protein concentrations were 0.53 g/l for control subjects, 0.67 g/l for CF heterozygotes and 0.86 g/l for CF homozygotes, respectively. By means of a Receiver Operating Characteristic (ROC) curve, a ‘CF homozygote diagnostic’ cut-off point was established at a salivary protein concentration of at least 0.86 g/l. From figure 10.1, it can be observed that the sensitivity of this ‘test’ was 56%, while the specificity was 89%. The area under the curve was 0.775.

10.5.2. SDS-PAGE results

Three distinct protein bands were considered relevant in this investigation. They were named band 1, 2 and 3. It was observed that bands 2 and 3 not only appeared as singlets, but also as doublets and triplets, depending on the study group (figure 10.2.). The molecular mass of these three proteins bands (singlet bands only) was
determined by means of linear regression analysis, with a standardized protein mix as reference.

Figure 10.1: Receiver Operating Characteristic (ROC) curve with the total salivary protein concentration as a diagnostic tool to distinguish CF homozygotes from CF heterozygotes and healthy subjects.
The respective molecular masses were 242 kDa (band 1), 161.5 kDa (band 2) and 68 kDa (band 3).

There was no significant difference between the three groups, regarding protein bands 1 and 2. It was, however, found that band 3 (singlet appearance) was significantly more often observed in CF homozygotes than in both other groups (P = 0.036). When this band appeared as two separate lines, it was found to appear borderline insignificantly more often in control subjects (P = 0.055). When this band appeared as a triplet it was significantly more often found in CF heterozygotes (P = 0.038).

**Figure 10.2.** Coomassie Brilliant Blue R250 stained SDS-PAGE gel with the standardized protein mix (position 1) and salivary samples of CF homozygotes (positions 3, 4, 9 and 10), CF heterozygotes (positions 5 and 6) and healthy control subjects (positions 7 and 8)
A multiple regression analysis model, with CF homozygotes as dependent variable and total salivary protein concentration and protein band 3 (singlet) as independent variables, was obtained with a predictibility of 39.5% and a correlation coefficient of 0.63 (P = 0.009).

10.6. Discussion

The present investigation confirmed earlier studies reporting that CF homozygotes had a significant higher total salivary protein concentration [Van Nieuw Amerongen, 1988a,b] than CF heterozygotes and healthy controls. However, the differences between CF heterozygotes and healthy controls were never shown before. To the knowledge of the investigator, this was one of the first studies ever in which salivary proteins of both CF heterozygotes and healthy controls were compared. As phenotypically CF heterozygotes can not be distinguished from the general population, this technique offers new perspectives, which should be explored into detail in the future on a larger scale; preferably in a multi centre study in which also different genotypes should be investigated.

It was interesting to find differences in protein bands between CF homozygotes, CF heterozygotes and healthy controls. However, the identification of the protein bands is therefore necessary, in order to provide more information on the salivary mechanisms at play in oral health in CF homozygotes. Consequently, relevant salivary protein interactions could be studied. As pointed out before, dental decay is the result of a multifactorial process. The least understood confounding factor is probably saliva as it is a mixture of cellular, organic and inorganic components.

Although the number of samples was limited, the multiple regression model which was obtained also offered new possibilities for identification of CF homozygotes and CF heterozygotes. It remains to be proven whether this model still holds when larger populations are studied.
10.7. Conclusion

The total salivary protein concentration and in particular a low molecular mass protein of 68 kDa, can be used to distinguish CF homozygotes from others. The identification of this protein is necessary in order to obtain better knowledge on the salivary mechanisms at play in CF homozygotes and non-CF homozygotes. The latter could imply a new perception in the mechanisms that are important in the development of dental decay.
GENERAL DISCUSSION AND CONCLUSION
Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited disease in Caucasians. It has a prevalence of about 1 in every 2000 live births and it is estimated that about 4 to 5 percent of Caucasians is heterozygous for the cystic fibrosis transmembrane regulator gene (CFTR), which is located on the long arm of chromosome 7 at position q31.

This single gene defect affects all exocrine glands and as a consequence, these glands produce a thicker viscous secretion than under healthy conditions. CF patients suffer from pancreatic insufficiency and recurrent pulmonary exacerbations, which means that they have to use antibiotics, mucolytica and expectorantia regularly and pancreas enzymes on a daily basis. Because of their disease, they can be considered vulnerable to develop dental decay, as they have to consume many in-between-meal-snacks and drinks, which are sugar rich. On the other hand, the medication can also be considered potentially dangerous to develop caries, as sugar containing syrups and beta-2 agonist containing aerosols are in most cases used at night, before going to sleep. Both were described to enhance enamel demineralisation indirectly.

Since the 1980’s it was well known that children with CF had significantly lower caries experience than healthy control subjects. Recently, however, a report was published on CF adolescents with a high caries experience. As no studies were available since the 1980’s on CF and oral health, a need for an updated study was felt. The null hypothesis of this study was that **CF homozygotes are potentially at risk for oral health and that saliva can be used for its determination.**

The aims of the study were:

1. Determination of the caries experience and oral cleanliness of CF homozygotes, CF heterozygotes and healthy controls.
2. Quantification of the oral bacterial flora with respect to caries in CF homozygotes, CF heterozygotes and healthy control subjects.

3. Assessment of the impact of medication, dietary habits and influence of oral hygiene habits on oral health in CF homozygotes, CF heterozygotes and control subjects.

4. Evaluation of salivary electrolyte concentration differences and differences in salivary proteins among CF homozygotes and between CF homozygotes, CF heterozygotes and healthy individuals.

5. Assessing the possibility to use human saliva as a diagnostic tool for the determination of oral health.

In a first approach (which corresponds with the first aim of this study), a matched case-control pilot study was carried out (chapter 2), in which the caries experience (DMF) of 37 healthy control subjects and 37 CF homozygotes was compared. There were no significant differences in caries experience found between these two groups. This was different from the results published in earlier literature on oral health and cystic fibrosis. Three hypotheses were brought forward, which could serve as possible explanation; geographical differences (e.g. environmental and genetical differences), the broader use of antibiotics (in the healthy control group also) and medical care differences (due to a better medical knowledge and understanding of the disease). Therefore the study needed to be enlarged and as today’s diagnostic tools have improved, CF heterozygotes were considered as a separate study group. The latter was never performed in the past. Moreover, CF heterozygotes were probably accidentally considered as healthy controls in the past.

A total number of 42 CF homozygotes, 48 CF heterozygotes and 62 healthy control subjects were studied in the further investigation. All CF heterozygotes were contacted via the CF homozygotes. Due to this way of subject selection,
confounding factors, such as socio-economical status, family, geographical spread and educational level, were excluded. It was obvious, however, that the mean age of the CF heterozygote group was considerably higher than that of the CF homozygote group. The latter implied that age remained the only confounding factor of influence. Consequently, it should be stressed that the results have to be interpreted with care. Additionally, the investigator is well aware of the rather small numbers of investigated subjects. However, this CF homozygote sample can be considered representative for the Belgian CF population, as it represents 7% of the total, which is in accordance with epidemiological standards.

In chapter 5 oral health (caries experience, dental plaque, calculus and gingival bleeding) was compared between 42 CF homozygotes, 48 CF heterozygotes and 62 healthy controls. CF homozygotes had a significant lower caries experience and less gingival bleeding sites than healthy controls and CF heterozygotes. CF heterozygotes showed a significant worse caries experience than CF homozygotes and healthy controls. These results called for a deeper investigation (as suggested in the aims of the study) in the determining factors of oral health in these three study groups.

Chapter 6 focussed on the caries relevant oral microbial situation (Mutans streptococci and Lactobacilli) in CF homozygotes, heterozygotes and healthy controls, by means of commercially available chair sid tests. It was found that CF homozygotes had significantly less high Mutans streptococci counts than both other groups. This corresponded with and reflected the lower caries experience found in CF homozygotes (chapter 5). However, the cause of these observations was not clear, as oral health is determined by many parameters, such as medication use, dietary habits, oral hygiene habits and salivary properties (organic and inorganic).

In chapter 7 medication and dietary habits were investigated (third aim). It became clear that medication (antibiotics, aerosols, pancreas enzymes and vitamins taken as maintenance ration) did not seem to play a major role in the caries
experience of CF homozygotes. It was found that CF homozygotes who opened their pancreas enzyme capsules, apparently did experience less dental calculus than those who did not. Dietary habits appeared to be only slightly different between the three groups in this study. Significantly more CF homozygotes consumed dairy products during meals and in between meals. Often in combination with a sweetened substance, which they need to provide them of the necessary body energy. This higher consumption of dairy products could partly serve as explanation for the lower caries experience (chapter 5) and the lower Mutans streptococci counts in CF homozygotes (chapter 6).

Subsequently, the oral hygiene habits and their impact on oral health of the three study groups were investigated (chapter 8). There were no differences in oral hygiene habits between the three study groups. Significantly more CF homozygotes than CF heterozygotes and healthy controls appeared to have received fluoride supplements. The latter was, however, not correlated with their lower caries experience.

None of the above mentioned parameters solely could sufficiently explain the lower caries experience in CF homozygotes. The latter once again supports the multifactorial aspects of the caries process. Subsequently, salivary electrolyte and protein concentrations were assessed (aims 4 and 5).

Primo, salivary electrolyte concentrations (sodium, potassium, chloride, bicarbonate, calcium and phosphate) were compared between the three study groups (chapter 9). Besides several differences between the several CFTR genotypes, major salivary electrolyte concentration differences were found between the three groups. It was shown that CF homozygotes and heterozygotes had a significant higher salivary sodium concentration than healthy controls. CF homozygotes also had a significant higher salivary phosphate concentration than CF heterozygotes and healthy controls. These findings could partly explain the lower caries experience in CF homozygotes, as the increased salivary sodium
concentration, which enhances enamel demineralisation, is possibly countered by an increased salivary phosphate concentration. The latter could also explain partly the higher caries experience in CF heterozygotes, as their increased salivary sodium concentration is not buffered by an increased salivary phosphate concentration. It was suggested that these distinct differences in salivary electrolyte concentrations may play an important role in the oral health status of any subject.

Secundo, the salivary protein composition (chapter 10) was investigated. The total salivary protein concentration was also significantly higher in CF homozygotes (0.86g/l) than in CF heterozygotes (0.67g/l) and healthy controls (0.56g/l). The use of the total salivary protein concentration as a diagnostic tool to differentiate CF homozygotes from the rest, was found to have a specificity of 89% and a sensitivity of 56%. It was concluded that this test might serve as additional test in the diagnosis of CF in newborns suspected of being CF patients.

It was also found that by means of sodium-dodecyl-sulphate poly acrylamide gel electrophoresis (SDS-PAGE) a 68 kDa salivary protein could be detected, which was representative for CF homozygotes. It remains to be explored whether this finding can be used as a diagnostic tool to detect CF homozygotes. Therefore further investigation is needed on a larger sample and to identify this protein (e.g. by amino acid sequencing, which is now possible as the human genome pattern was recently discovered).

It became clear from chapter 9 and 10 that saliva indeed can be used for diagnostic purposes. However, there is still one unidentified feature dealing with the typical clinically visible increased salivary viscosity in CF homozygotes. Salivary viscosity is caused both by salivary proteins and the cellular content of saliva (epithelial cells, erythrocytes, leukocytes). In order to study the salivary protein concentration, saliva had to be centrifuged, which means that “heavy” material is deposited at the bottom of the test tube (the greater the g-force, the more
particles will be deposited in the test tube), which in its turn results in a loss of viscosity, as the supernatant contains only little amounts of “large” particles.

In a further attempt to quantify viscosity, the cellular content of human saliva was measured by means of flow cytometry and reference values were established in healthy individuals (see Appendix). This spin-off investigation opened new possibilities and perspectives to use salivary flow cytometry to assess oropharyngeal infections and to assess cellular content changes in saliva. Actually reference values in healthy control subjects were established. Further investigation in medically compromised patients will show the possibilities of this innovative technique.

In conclusion it can be stated that, although CF patients were considered potentially to have a high risk caries profile, they had a significant lower caries experience than both CF heterozygotes and healthy controls. This was in disagreement with the null hypothesis.

On the other hand, the salivary composition, both inorganic (sodium and phosphate) and organic (proteins), was found to be significantly different between the three study groups. This means that the use of saliva, as a diagnostic tool, in the determination of oral health can be of significance.

It remains to be clarified, whether, CF homozygotes possess “intrinsic” (inherent to their disease) salivary compensatory mechanisms against dental decay.
Algemene discussie en conclusie

(General discussion and conclusion in Dutch)
Mucoviscidose (MV) is de meest voorkomende dodelijke recessief overerfbare aandoening in het blanke ras. Ongeveer 1 op elke 2000 levend geboren is een MV patiëntje en 4 à 5 procent van de blanke bevolking zou drager zijn van het « cystic fibrosis transmembrane regulator » (CFTR) gen, welk gelokaliseerd is op de lange arm van chromosoom 7, op positie q31.


Studies uit de jaren 80 vermelden een lager cariës voorkomen bij kinderen met MV dan bij gezonde kinderen. Maar in een recente publicatie werd het tegenovergestelde gerapporteerd bij adolescenten met MV. Bij gebrek aan recente studies (de recentste dateren van de jaren 80) over de orale gezondheid bij MV patiënten, werd de nood aangevoeld een « up-to-date » onderzoek op te starten. De nul hypothese van dit onderzoek was dat MV patiënten een potentieel risico lopen op een slechte orale gezondheid en dat speeksel kan gebruikt worden om dit te bepalen.

De doelstellingen van het onderzoek waren;
1. Het bepalen van de status praesens van het cariës voorkomen en de orale gezondheid bij mucoviscidose patiënten, muco-heterozygoten en gezonde controlepersonen.

2. Het kwantificeren van de orale cariogene bacteriële flora bij mucoviscidose patiënten, muco-heterozygoten en gezonde controlepersonen.

3. Het bepalen van de impact van medicatie, dieetgewoonten en mondhygiëngewoonten op de orale gezondheid van mucoviscidose patiënten, muco-heterozygoten en gezonde controlepersonen.

4. Het bepalen van verschillen in de concentratie van speeksel electrolieten en speekseleiwitten tussen mucoviscidose patiënten, muco-heterozygoten en gezonde controlepersonen.

5. Het toetsen van de diagnostische mogelijkheden van speeksel in de bepaling van de orale gezondheid.

In eerste instantie werd een piloot studie opgestart met 37 MV patiënten en 37 gezonde controle personen (hoofdstuk 2) bij dewelke het cariësvoorkomen (DMF) werd vergeleken (conform de eerste doelstelling van dit onderzoek). Er werden echter geen significante verschillen gevonden tussen beide groepen. Dit was opvallend verschillend met de bestaande literatuur over orale gezondheid en MV. Drie hypothesen werden naar voren geschoven als mogelijke verklaring voor deze vondst; geografische verschillen (bijv. omgevingsfactoren en genetische variaties), het veralgemeender gebruik van antibiotica (ook in de gezonde populatie) en een verschil in medische verzorging (dankzij de verbeterde inzichten en kennis van de ziekte). Daarom werd geopteerd om de studiegroep uit te breiden en daarenboven, werden, gezien de actuele diagnostische mogelijkheden, de MV heterozygoten als een aparte groep te beschouwd. Waarschijnlijk zijn er zelfs, door onwetendheid, in het verleden MV heterozygoten als controlepersonen gebruikt geweest.
In het totaal werden 42 MV patiënten, 48 MV dragers en 62 controlepersonen in het verdere onderzoek betrokken. Alle MV heterozygoten werden geselecteerd via de MV homozygoten. Op deze manier konden socio-economische status, familiale samenstelling, geografische spreiding en opleidingsniveau, worden uitgeschakeld als invloedrijke factoren. Het is dan ook vanzelfsprekend dat de gemiddelde leeftijd van de MV heterozygoten aanzienlijk hoger was dan die van de MV patiënten groep. Dit betekent wél dat leeftijd de enig invloedrijke factor blijft, die ervoor zorgt dat de resultaten van dit onderzoek met de nodige voorzichtigheid moeten geïnterpreteerd worden. Daarenboven wenst de auteur duidelijk te maken dat hij er zich van bewust is dat het om kleine onderzoeksgroepen gaat, maar dat de MV patiënten groep wel degelijk als representatief voor de Belgische MV populatie kan beschouwd worden, omdat ze 7% uitmaakt van het totaal, welke beantwoord aan epidemiologische criteria.

In hoofdstuk 5 werd de orale gezondheid (cariës voorkomen, tandplaque, tandsteen en gingivale bloedingen) van 42 MV homozygoten, 48 MV heterozygoten en 62 gezonde controlepersonen onderzocht. MV homozygoten hadden een significant lagere DMF (-T en -S) score en significant minder tanden met bloedend tandvlees dan MV heterozygoten en gezonde controlepersonen. MV heterozygoten hadden significant meer tanden verloren dan controlepersonen. Hun DMF was net niet significant hoger dan die van de controlegroep. Deze resultaten eisten een dieper onderzoek naar de determinanten van de orale gezondheid in deze drie onderzoeksgroepen (zoals gesuggereerd in de doelstellingen).

In hoofdstuk 6 werd de cariës relevante orale microflora, door middel van commercieel verkrijgbare “chair-side testen”, bestudeerd (*Streptococcus mutans en Lactobacillen*) tussen MV homozygoten, heterozygoten en controlepersonen. MV patiënten hadden significant minder *Streptococcus mutans* kolonies dan beide andere onderzoeksgroepen. Dit resultaat confirmeerde én weerspiegelde tevens het lagere cariës voorkomen bij MV patiënten (*hoofdstuk 5*). Het dient opgemerkt te
worden dat de oorzaak van deze bevindingen niet duidelijk is, omdat orale gezondheid door een waaiervan parameters, zoals medicatie gebruik, voedingsgewoonten, mondhygiëne en speekselsamenstelling (organisch én anorganisch), wordt beïnvloed.

In hoofdstuk 7 werden zowel de medicatie alsook de voedingsgewoonten (de derde doelstelling) bestudeerd. Medicatie (patiënten met een onderhoudsdosis van antibiotica, aërosolen, pancreas enzymes en vitaminepreparaten) bleek een minder belangrijke impact te hebben op de DMF score van MV homozygoten. Er werd vastgesteld dat de MV patiënten die hun pancreasenzyme capsules openden, significant minder tandsteen hadden dan diegenen die het niet deden. Wat de dieetgewoonten betreft, werden slechts kleine verschillen aangetroffen tussen de drie onderzoeksgroepen. Significant meer MV patiënten gebruikten melkproducten tijdens maaltijden en tussendoortjes, meestal zelfs in combinatie met suikerrijke bestanddelen, welke ze nodig hebben om zich dagelijks van de nodige lichaamsenergie te voorzien. Het aspect van de melkproducten kan als gedeeltelijke verklaring dienen waarom MV patiënten significant lagere DMF scores hadden en significant minder *Streptococcus mutans* kolonies in hun speeksel vertoonden (hoofdstuk 6).

In hoofdstuk 8 werden de mondhygiënegewoonten bestudeerd tussen de drie onderzoeksgroepen, maar er bleken geen significante verschillen te zijn. Er waren wel significant meer MV homozygoten dan MV heterozygoten en controlepersonen die fluoridesupplementen hadden genomen. Dit bleek echter niet gecorreleerd te zijn met de DMF waarde bij MV patiënten.

Uit het voorgaande blijkt dat geen enkele van de hierboven onderzochte parameters de orale gezondheid van de drie onderzoeksgroepen kunnen verklaren. Dit bevestigd het multifactoriële aspect van het cariësproces. Vervolgens werden zowel de ionaire samenstelling alsook de eiwitsamenstelling van het speeksel onderzocht (doelstelling 4 en 5).
In hoofdstuk 9 werden verschillen in speekselelectrolieten (natrium, kalium, chloor, bicarbonaat, calcium en fosfaat) tussen de drie onderzoeksgroepen bestudeerd. Naast het feit dat er verschillen in electrolietensamenstelling werden gevonden tussen de verschillende types van CFTR genotypes, bleken er ook twee duidelijk significante verschillen te bestaan tussen MV homozygoten en heterozygoten. MV homozygoten en heterozygoten hadden significant hogere natriumconcentraties in hun speeksel dan controlepersonen. Tegelijk hadden de MV patiënten ook een significant hogere fosfaatconcentratie in hun speeksel dan de MV heterozygoten en controlepersonen. Deze bevindingen kunnen op hun beurt deels verklaren waarom MV patiënten significant lagere DMF scores hadden; een hoge natriumconcentratie in het speeksel, die glazuur demineralisatie in de hand werkt, kan worden tegengewerkt door de hogere fosfaatconcentratie. Omdat ze geen compenserende (hogere) fosfaatconcentratie vertoonden in hun speeksel, kan dit tevens verklaren waarom MV heterozygoten méér tandbederf vertoonden.

Daarop volgend werden in hoofdstuk 10 verschillen in speekseleiwitten tussen de drie groepen bestudeerd. De totale speeksel eiwitconcentratie bij MV patiënten (0.86g/l) was significant hoger dan die van MV dragers (0.67g/l) en van controlepersonen (0.56g/l). Het gebruik van de totale speeksel eiwitconcentratie als diagnostisch criterium om MV homozygoten te kunnen onderscheiden van de rest, bleek een specificiteit te hebben van 89% en een sensitiviteit van 56%. Er werd besloten dat deze test eventueel als aanvullende test zou kunnen dienen bij de diagnose van MV bij pasgeboren met een vermoeden van MV. Door middel van «sodium-dodecyl-sulphate-polyacryl amide gel electrophoresis» (SDS-PAGE) werd een eiwit met een molecuulgewicht van 68 kDa geïdentificeerd, dat representatief was voor MV patiënten. Of deze techniek kan gebruikt worden als diagnostische techniek moet nog verder onderzocht worden. Hiervoor is uiteraard verder onderzoek nodig op grotere schaal en is het ook noodzakelijk om het eiwit te identificeren (bijv. door eindstandige aminozuursequenties te gaan.
bepalen, welke vandaag de dag mogelijk is, sinds de ontdekking van het menselijk genoom).

Het is duidelijk uit hoofdstukken 9 en 10 dat speeksel wel degelijk diagnostische eigenschappen heeft. Er blijft echter nog één aspect over, namelijk de klinisch waarneembare verhoogde speekselviscositeit van MV patiënten. Speekselviscositeit wordt bepaald door de eiwitten enerzijds en door de cellulaire inhoud (epitheelcellen, erythrocyten en leukocyten) van het speeksel anderzijds. Om de totale eiwitconcentratie te bepalen, moet speeksel gecentrifugeerd worden, wat betekent dat de “zware” deeltjes naar de bodem van de proefbuis zullen zinken (hoe groter de g-krachten, hoe meer partikels op de bodem van de proefbuis zullen neerdalen). Dit resulteert in een verlies van viscositeit omdat het supernatans bijna geen “grote” partikels zal bevatten.

In een poging om de viscositeit te kwantificeren, werd de cellulaire samenstelling van speeksel bestudeerd aan de hand van een flow cytometer en werden er referentiewaarden opgesteld van gezonde personen (zie Appendix). Dit spin-off onderzoek opende nieuwe mogelijkheden en perspectieven om speeksel flow cytometrie te gebruiken om oro-faryngeale infecties op te sporen en om de cellulaire inhoud van speeksel te gaan bepalen. Tot op heden werden enkel nog maar referentiewaarden voor gezonde personen opgesteld. Verder onderzoek bij medisch gecompromitteerde patiënten zal de mogelijkheden van deze nieuwe techniek bepalen.
Als algemene conclusie van dit proefschrift kan worden gesteld dat ondanks het feit dat MV patiënten kunnen beschouwd worden als individuen met een hoog cariës risico profiel, zij significant lagere DMF scores en een betere orale gezondheid bleken te hebben dan MV heterozygoten en gezonde controlepersonen. Dit was in tegenspraak met de nulhypothese.

Wat het speeksel betreft, bleek dat zowel de anorganische (natrium en fosfaat concentraties) als de organische (totale eiwitconcentratie) samenstelling significant verschillend waren tussen de drie onderzoeksgroepen. Hieruit kan besloten worden dat het gebruik van speeksel, als diagnostisch middel voor het bepalen van mondgezondheid, van betekenis kan zijn.

Het blijft echter onvoldoende bewezen of MV patiënten inderdaad beschikken over een soort van «intrinsiek» (inherent aan hun ziekte) compensatoir speekselmechanisme dat hen in zeker zin beschermt tegen tandbederf. Hiervoor is diepgaander vervolgonderzoek nodig.
Flow Cytometry as a New Method to
Quantify the Cellular Content of
Human Saliva and its Relation to Gingivitis

This chapter was published as:
During the clinical examinations, it was observed that very often CF homozygote saliva samples could be distinguished from CF heterozygote and control samples, due to their high viscosity. By means of capillary viscosimetry (PVS1 LAUDA$^\text{®}$), an attempt was made to measure salivary viscosity. It was observed that clinically viscous saliva samples “lost” their viscosity when centrifuged and that the clinically visible difference disappeared [Van den Maagdenberg, 2000].

It should however be emphasized that saliva behaves like an emulsion and that the expression “viscosity” is not entirely correct, as Newton’s law is not applicable on these kind of biological fluids. Saliva should be considered as a gel, a colloidal system, which includes that by homogenizing the sample or by applying forces on the sample, the internal structure is ruptured. Consequently, the measured viscosity does not match the “actual” one. The latter implies that besides proteins, several particles, such as bacteria, epithelial cells, leukocytes and erythrocytes, strongly influence salivary rheology [Saunders, 1971; Blair, 1974; Van der Reijden, 1993; De Smedt, 1995; Glantz et al., 1995].

Due to the above mentioned considerations and especially the fact that rheological properties of saliva are influenced by the cellular content of the sample, which probably reflects oral health, the next study using flow cytometry was carried out to find out if this cellular content could be measured.

As this technique had never been used for saliva before, it was necessary, as recommended by the Internation Federation of Clinical Chemistry to establish reference values first from healthy individuals.
ABSTRACT

Determining the cellular content of saliva by means of conventional microscopy chamber counting is a very time consuming and operator sensitive procedure. This study concentrated on the use of flow cytometry to examine the cellular content of saliva. Erythrocytes, leukocytes, epithelial cells and bacteria were quantified and the results were compared with caries experience and the presence of gingivitis.

Two hundred and fifty eight uncentrifuged vortexed paraffin stimulated saliva samples (112 males and 146 females) were analyzed with the UF-100® flow cytometer. Salivary reference values were established for erythrocyte, leukocyte, epithelial cell and bacterial count. Caries experience (DMF) and the presence of gingivitis were recorded.

Caries experience or caries risk could not be assessed with flow cytometry. However, salivary flow cytometry may be useful in determining an individual’s risk for gingivitis: a significant increase in salivary leukocytes was observed in individuals with gingivitis. At a cut-off level of 10³ leukocytes µL⁻¹ saliva, a sensitivity of 76% and a specificity of 45% was obtained. Other analytes were not significantly different between individuals with and without gingivitis.

Flow cytometry of paraffin stimulated human saliva seems a promising diagnostic or predictive tool and further investigations of diseases of the oro-pharyngeal loge, such as tonsillitis and periodontitis, should be carried out in the future.
A.1. Introduction

Whole human saliva is a complex exocrine secretion which contains proteins, ions, bacteria and cells derived from salivary glands, crevicular fluid, tonsils, the oral mucosa, bronchopulmonar and oropharyngeal secretions [Van Nieuw Amerongen, 1988a; Edgar and O’Mullane, 1990; Mandel, 1990; Schonfeld, 1992; Smith, 1992; Ten Cate, 1994; Schenkels et al., 1995; Burkitt et al., 1997; Marsh and Martin, 1999a,b]. Despite the fact that a lot of research has been carried out on the chemical composition of saliva, little is known about its cellular components.

Ongoing local inflammations can be investigated by means of flow cytometry of human body fluids. Flow cytometry allows a fast and accurate determination of epithelial cells, erythrocytes, leukocytes and bacteria in human secretions, such as urine [Ben-Ezra et al., 1998; Kouri et al., 1999; Langlois et al., 1999; Lun et al., 1999], cerebrospinal fluid [Ziebig et al., 2000; Van Acker et al., 2001] and sperm [Muylaert et al., 2000].

Until now, no flow cytometry studies on saliva have been performed. Due to this the aims of the present study were both analytical and clinical. Quantification of erythrocytes, leukocytes, epithelial cells and bacteria in paraffin stimulated whole human saliva was carried out by means of flow cytometry. Investigation of the reproducibility of saliva flow cytometry was also performed. Furthermore reference values for these parameters in saliva were established. Next to these analytical aims, possibilities of using flow cytometry to trace gingival inflammation by a non-operator-sensitive technique, which avoids probing of the gingival tissues were explored. Finally assessment of caries experience (DMF) by means of saliva flow cytometry was also investigated.
A.2. Material and methods

A.2.1 Patients

Subjects suffering from cardiovascular, endocrine, hematological, infectious or genito-urinary diseases were excluded from the study. A sample of 258 healthy volunteers (112 males and 146 females, mean age 33 ± 13 years) was selected. Every subject signed an informed consent and the study was approved by the University of Ghent Hospital’s ethical committee.

A.2.2. Oro-dental data

The investigator (J.A.) was specifically trained at baseline and participated both in an in vitro and in vivo calibration exercise, according to the British Association for the Study of Community Dentistry (BASCD) guidelines [Pine et al., 1997]. The investigator’s overall kappa values for both exercises were respectively 0.84 and 0.76, both standing for an acceptable interreliability with an experienced examiner (L.M.) as a benchmark [Bolin et al., 1995]. The caries experience index (DMF-T and DMF-S) of 213 of the 258 volunteers (82.6%) was determined. This was performed according to the WHO report on oral health surveys (white light source, dental mirror, CPITN probe Type E and cotton rolls) [http://www.whocollab.od.mah.se] and based upon the diagnostic criteria for caries prevalence surveys, published by the BASCD [Pitts et al., 1997]. Caries was recorded at the level of cavitation. No X-rays were taken. The abbreviations « D, M and F » respectively stand for « decayed, missing (extracted due to decay) and filled » teeth (index –T) or surfaces (index –S). Gingivitis was recorded when at least one hypertrophic and hyperaemic gingival margin was present. As dichotomized data were preferable for statistical analysis, no specific index was used to express the severeness of gingivitis, as this was not relevant regarding the aims of this study.
A.2.3. Saliva sampling

All subjects were asked to chew a paraffin tablet, as provided in the commercial available CRT® test kit (Vivadent®, Liechtenstein), for at least 3 minutes. The secreted saliva was collected in a sterile plastic recipient and transported to the laboratory at a temperature of 18°C. All subjects were in an upright position when saliva was collected and the collections were performed between 9 a.m. and 4 p.m. in the presence of the investigator.

A.2.4. Saliva flow cytometry

The Sysmex UF100® (TOA Medical Instruments Co, Kobe, Japan) was used to determine the cellular composition. The Sysmex UF100® uses an argon laser to analyse the electrical impedance for volume, for forward light scatter and for size. DNA and membranes are analyzed, respectively with phenanthridine and carbocyanine, by fluorescent dyes. The pulse intensity and width of the forward scattered light and fluorescence light are measured. These data, combined with the impedance data of the formed elements, are categorized by multiparametric algorithms based upon their size [Sysmex UF-100 manual, 1995]. The instrument aspirates 800µL of the sample and the results are displayed as scatter grams and as counts per microliter. For this study saliva was not centrifuged, as it was the purpose to quantify the cellular content of saliva. Instead, it was vortexed for at least 20 seconds, before analysis with the Sysmex UF-100® was performed. The analysis was performed at room temperature, within 1 hour after collection.

A.2.5. Method validation

As the presented technique was never performed on saliva, it was necessary to investigate the reproducibility and the influence of sample storage (temperature and time) on the analysis results.
A.2.5.a Reproducibility

Three adult healthy volunteers provided each a paraffin stimulated saliva sample, which was analysed 10 times in the UF-100® flow cytometer. The within run coefficient of variation (CV) was determined by means of the Medcalc® (Medcalc® software – Mariakerke, Belgium) statistical software program.

A.2.5.b Variation of cellular content during the day

Five healthy adult volunteers provided each at 4 different times of the day (9 a.m., 11 a.m., 2 p.m. and 4 p.m.) a paraffin stimulated saliva sample. Immediately after collection the samples were analysed in the UF-100®. The latter was performed to obtain an impression of the intra- and interindividual fluctuations of the cellular content of saliva. Subsequently the between run coefficient of variation (CV) was determined, using the Medcalc® program.

A.2.5.c Influence of storage temperature and time

Six paraffin stimulated saliva samples were obtained from six healthy adult volunteers at 9 a.m. The samples were subsequently divided into 2 equal volumes and stored at 6 °C and at room temperature (20 °C). Every sample was subsequently measured in the UF-100® flow cytometer at 9.30 a.m. (= baseline measurement), 11 a.m., 2 p.m. and 4 p.m.

A.3. Statistics

All data were immediately stored in an automated database and appropriate statistical analysis was performed, using the Medcalc® program (Medcalc® Software – Mariakerke, Belgium). Within-run imprecision and interindividual variations were expressed as coefficient of variation (CV). Reference values for the various parameters were calculated according to the IFCC (International Federation of Clinical Chemistry) guidelines [Sasse et al., 1995]. Statistical differences were
evaluated using the unpaired Wilcoxon test. A P-value < 0.05 was considered as statistically significant. The statistical differences between samples stored at 6°C and 20°C were calculated by means of a paired Wilcoxon signed ranks test. A Box-and-Whisker plot (figure 1) and a ROC (Receiver Operating Characteristic) curve analysis (figure 2) were used to visualize the significant difference in leukocyte count between individuals with and without gingivitis [Metz, 1978].

A.4. Results
A.4.1. Method validation
A.4.1.a Reproducibility

Both the within-run as well as the between-run coefficient of variation (CV) of each analyte was carefully determined. The within-run CV for erythrocytes was 27%, 29% for leukocytes and epithelial cells and 24% for bacterial count. The between-run CV for erythrocytes was 49%, 47% for leukocytes, 29% for epithelial cells and 50% for bacterial count. The mean within-run CV and between-run CV were respectively 27 and 44%. This illustrates, with exception of epithelial cells, the broad range of cellular counts during the day.

A.4.1.b Storage time and temperature influences

No significant differences were found between the UF-100® erythrocyte and leukocyte readings for samples (n = 6) stored at room temperature (20°C) and refrigerator temperature (6°C). On the contrary, it was observed that the epithelial cell count and the bacteria count were both significantly higher (P < 0.01) when samples were stored for 7 hours at room temperature.
A.4.1.c Experimental sample evaluation

Clinical data

The prevalence of gingivitis in the study group was 25% (n = 54; 21 males and 33 females). The mean DMF-T and DMF-S were respectively 7.8 ± 6.7 and 23.1 ± 27.3.

Neither for gingivitis, nor for DMF, significant differences were observed between both sexes.

Table A.1: Reference values for salivary cells of paraffin stimulated saliva.

Figures for the overall sample and a comparison between males and females. ( ** P < 0.01, *** P < 0.001 )

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Over-all (n = 258)</th>
<th>Males (n = 112)</th>
<th>Females (n = 146)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median, 2.5th &amp; 97.5th percentile (µL⁻¹)</td>
<td>Median, 2.5th &amp; 97.5th percentile (µL⁻¹)</td>
<td>Median, 2.5th &amp; 97.5th percentile (µL⁻¹)</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>716 (123 – 3388)</td>
<td>918 *** (189 – 4244)</td>
<td>580 (116 – 3269)</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>1279 (257-5142)</td>
<td>1239 (299 – 5016)</td>
<td>1404 (237 – 5158)</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>935 (162 – 4191)</td>
<td>1156 *** (244 – 4703)</td>
<td>753 (136 – 2942)</td>
</tr>
<tr>
<td>Bacteria</td>
<td>11511 (3785 – 37249)</td>
<td>12748** (4024 – 43810)</td>
<td>10439 (3727 – 31465)</td>
</tr>
</tbody>
</table>

Flow cytometer reference values

In table 1 the UF-100® readings are shown for the study group (n = 258; 112 males and 146 females). This table depicts a summary of the distribution of erythrocytes, leukocytes, epithelial cells and bacteria for the overall sample and according to gender. The numbers are presented as medians and 2.5th and 97.5th percentiles.
percentiles. For all analytes studied, reference ranges are remarkably broad. For erythrocyte (P = 0.0002), epithelial (P = 0.0001) and bacterial cell counts (P = 0.0026), significant higher counts were observed in males.

**Figure A.1.** A Box-and-Whisker plot of the salivary leukocyte count (log leukocytes per microlitre) in the presence or absence of gingivitis.

A.4.1.d Clinical data and saliva flow cytometry

A significant difference (P = 0.042) in salivary leukocyte count was observed between individuals with (1881 ± 1349 cells µL\(^{-1}\)) and without gingivitis (1399 ± 993 cells µL\(^{-1}\)). A Box-and-Whisker plot shows the difference in leukocyte count (figure 11.1.).
Salivary leukocyte count was considered to have a potential predictability for an individual to have gingivitis, therefore a ROC curve analysis was implemented. At a cut-off level of $10^3$ leukocytes per microliter, a sensitivity of 90% and a specificity of 49.1% was obtained (Figure 11.2.). This was not observed for epithelial cells or bacteria or for erythrocytes.

From analogue analyses, with caries experience as a response variable, no significant differences were found.

**Figure A.2.** A receiver operating characteristic (ROC) curve of the salivary leukocyte counts (log leukocytes per microlitre) in case of gingivitis, with a diagnostic cut-off point at $10^3$ leukocytes per microlitre whole saliva, standing for a sensitivity of 90% and a specificity of 49.1%
A.5. Discussion

The reproducibility of paraffin stimulated whole human saliva flow cytometry was acceptable. The CVs for erythrocyte, leukocyte and epithelial cell count were higher than for bacterial count. Compared with literature [Ben-Ezra et al., 1998] on the use of the UF-100® instrument, these CVs are considerably higher than for urinalysis, which can be explained by the higher number of samples in the urine study and by the fact that the instrument is designed and calibrated to analyse urine and not saliva. The broad range in cellular counts was attributed to clustering of cells and to a broad range of inter-individual variation in salivary composition.

The broad range of flow cytometer counts during the day implicates that an individual’s flow cytometer saliva profile should not be based upon one measurement as large fluctuations occur during the day. The same holds for chemical analysis of saliva [Aguirre et al., 1993]. It is recommended that several salivary samples during the day should be taken for several consecutive days, to obtain an individual’s salivary flow cytometer profile. Analysis of only one sample is merely an instantaneous detection of an individual’s salivary cellular composition at a precise moment in time.

The significant higher bacterial count at room temperature was obviously caused by the higher bacterial activity and capacity to reproduce at room temperature, as food debris, epithelial cells and polysaccharide chains from salivary proteins serve as nutrients during storage [Van Nieuw Amerongen, 1988a; Rudney, 1995; Tenuvuo, 1998; Marsh and Martin, 1999a,b].

The significant higher epithelial cell count at room temperature was attributed to unraveling of clusters of epithelial cells at this temperature. At 6°C epithelial cell clusters will less likely fall apart. When the sample is « fresh », epithelial cells will adhere and the flow cytometer will detect clusters of cells. The latter results apparently in a lower epithelial cell count. When the sample is stored at 20°C, the clusters may disintegrate, and as a consequence, the flow cytometer will
«identify» more individual cells. This explains the higher number at room temperature. A similar finding was reported on urinalysis, where at 20°C also a higher count was detected in 1 of 16 specimens [Kouri et al., 1999].

Obviously no significant differences were found for erythrocyte and leukocyte counts which is in concordance with other authors [Kouri et al., 1999].

It is recommended to analyse salivary samples in the flow cytometer as soon as possible after collection or to store them at low temperature, if analysis cannot be performed immediately after sample collection. This advise is endorsed by publications on chemical and bacterial analyses of saliva [Davis et al., 1991; Schonfeld, 1992; Smith, 1992; Bollen et al., 1999; Marsh and Martin, 1999a].

Flow cytometry, by means of the Sysmex UF-100® flow cytometer, is known as a fast and reliable technique to analyze the cellular composition of a biological fluid [Ben-Ezra et al., 1998; Kouri et al., 1999; Lun et al., 1999]. Moreover, it is not operator sensitive as is conventional chamber counting [Kouri et al., 1999]. Salivary flow cytometry is now clearly demonstrated to be among the possible applications of the Sysmex UF-100®.

The observed significant higher erythrocyte, epithelial and bacterial cell counts in males in the experimental group were probably coincidental and should be interpreted with care. Gingivitis was associated with increased salivary leukocyte counts. Salivary leukocyte count may be useful as a biological marker for gingival inflammation. This is the first study that quantifies leukocytes in saliva to confirm the clinical presence of gingivitis. No literature data on salivary leukocyte counts and gingivitis are available.

As most of the salivary leukocytes are derived from the oropharyngeal area and from the crevicular sulcus [Ten Cate, 1994; Marsh and Martin, 1999a,b] , it should be emphasized that a high leukocyte count could also be related to other oropharyngeal infections, such as tonsillitis [Kirstila et al., 1996; Brook et al., 1997].
Although tonsillitis and other oropharyngeal infections were exclusion criteria in the present study, other causes of oropharyngeal inflammation could be considered as theoretical causes for an increased salivary leukocyte count.

No significant correlations between DMF-T or DMF-S and the flow cytometry data were observed. This can probably be explained by the fact that flow cytometry of saliva is a current recording of the cellular content of this secretion fluid on the one hand and that DMF is both a recording of the dental history (missed and filled teeth or surfaces) as well as a recording of the present dental situation (decayed teeth and surfaces) of an individual on the other hand.

It should also be emphasized that the UF-100® cannot distinguish cariogenic bacteria from other commensals or periodontal pathogens. The number of bacteria (both viable and non-viable) only represents the amount of cells, this analyzer can categorize as such. Therefore the UF-100® should not be used to assess an individual’s caries risk, by measuring the number of bacteria per microliter saliva.

A.6. Conclusion

The use of the Sysmex UF-100® flow cytometer is a fast and easy way to determine the cellular composition of saliva. Flow cytometry of saliva may be useful in assessing the presence of gingivitis in an individual. The salivary leukocyte count is a discriminative parameter for gingivitis.

Saliva flow cytometry should, however, not be used to evaluate the caries experience or to determine caries risk in individuals.

Further research is needed to determine and to evaluate other applications of salivary flow cytometry as a predictive and discriminative parameter for oral disease or infection, such as tonsillitis and periodontitis.

Acknowledgements
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