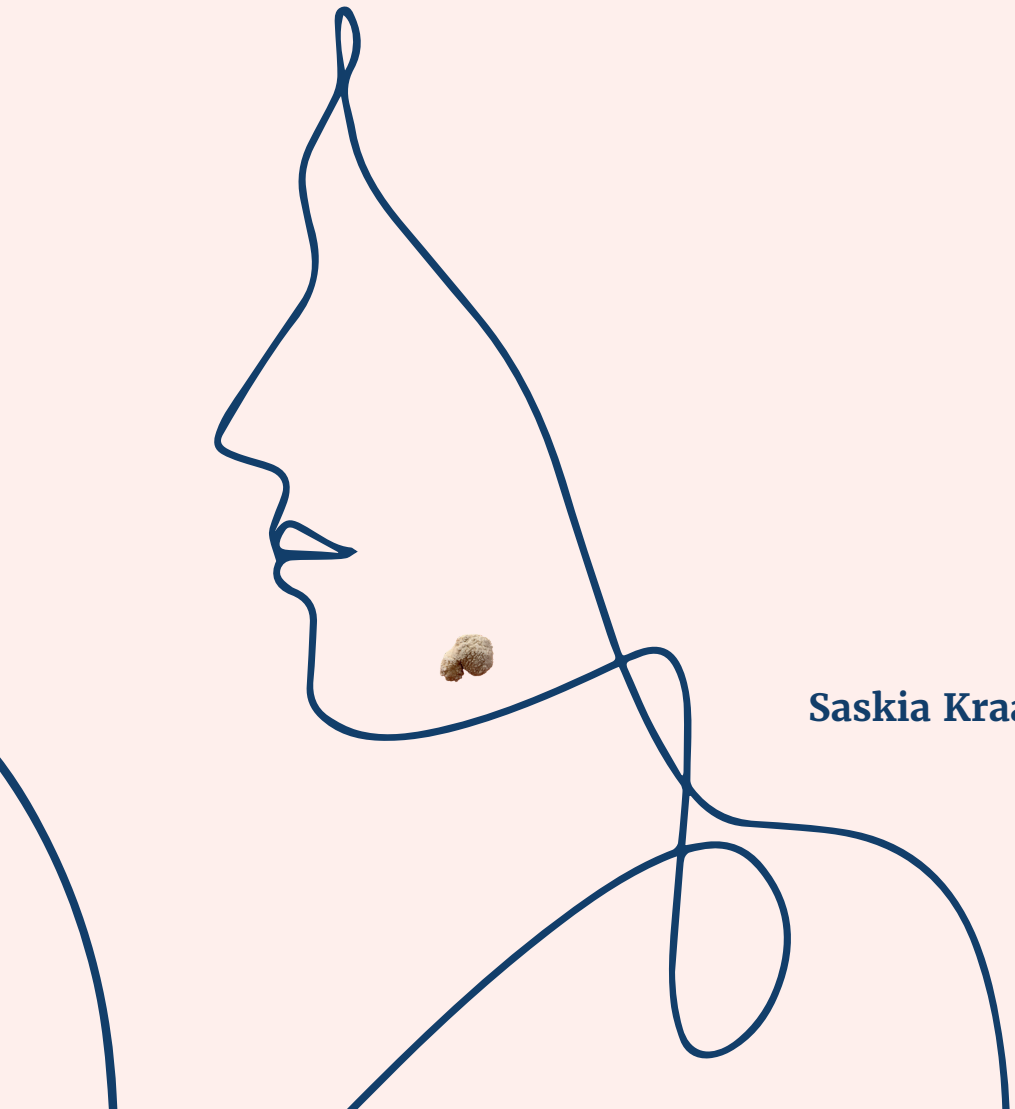
SALIVARY STONES

aspects of the composition, diagnosis and patient related factors



Saskia Kraaij

SALIVARY STONES:

ASPECTS OF THE COMPOSITION, DIAGNOSIS AND PATIENT RELATED FACTORS

Saskia Kraaij

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Salivary stones: aspects of the composition, diagnosis and patient related factors

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Saskia Kraaij

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promotoren:	prof.dr. J.G.A.M. de Visscher
	dr. H.S. Brand
copromotor:	prof.dr. F.J. Bikker
promotiecommissie:	prof.dr. A. Vissink
	prof.dr. T. Forouzanfar
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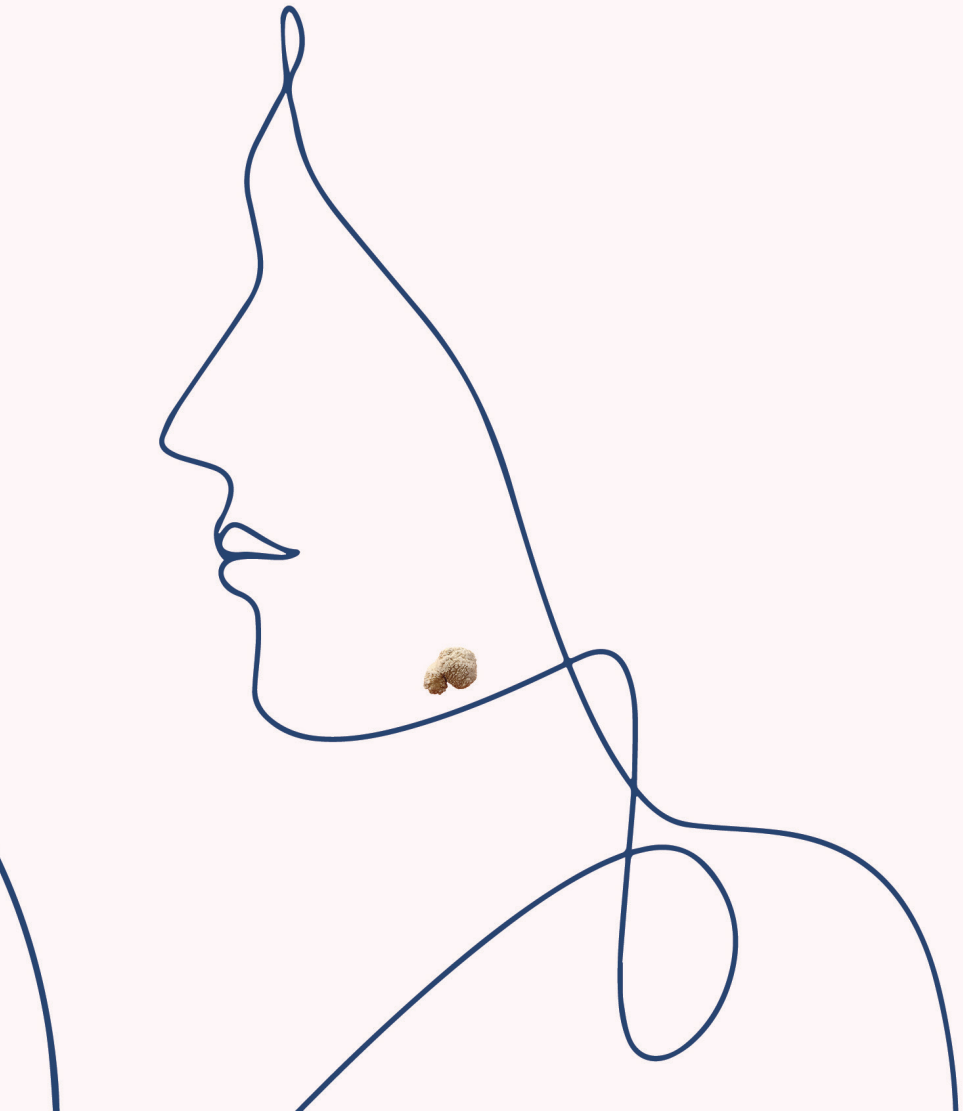

CHAPTER 1

General introduction

GENERAL INTRODUCTION

Saliva and salivary glands

Saliva is an important body fluid, which is of essential importance for the maintenance of oral health: it moistens and lubricates the oral mucosa, protects the teeth in various ways, contributes to taste and starts digestion of food (Figure 1). To perform all these functions, saliva contains a multitude of (glyco)proteins, minerals and other constituents (1, 2).

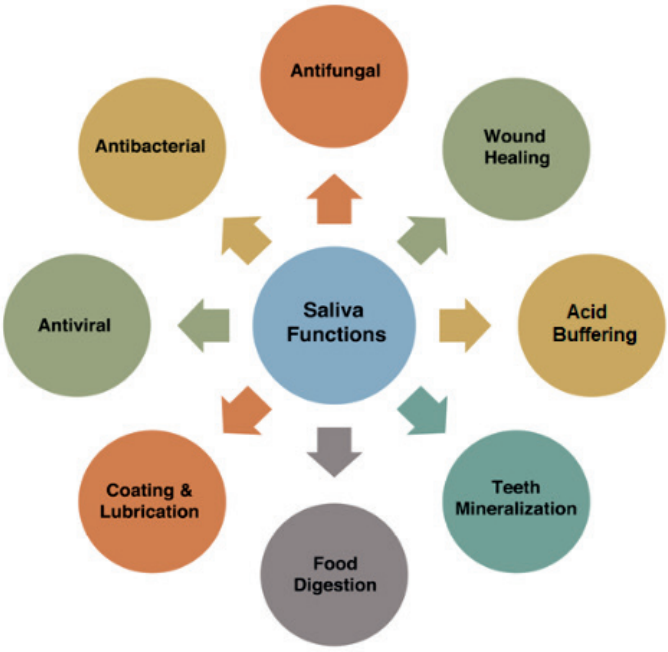

Figure 1 – An overview of the different functions of saliva. Modified from Vila et al (2).

In healthy adults, salivary flow rates range between 0.6 and 1.5 liter of saliva per day under non-stimulated conditions, which equals to an unstimulated salivary flowrate of 0.3-0.5 mL/min, during waking hours of the day (1, 3). Whereas during sleep salivary flow rates decrease to approximately 0,1 mL/min. Taste, scent and mechanical stimulation (chewing), will lead to an increased saliva secretion rate of up to 1.2 to 3.0 mL/min (4). The unstimulated whole saliva flow is a mixture of secretions and is mainly (90%) produced by three bilateral pairs of major salivary glands; the parotid glands (25%), the

submandibular glands (60%) and the sublingual glands (5%) (1) (Figure 2). The remaining 10% is produced by the 700-900 minor intraoral salivary glands. These are located on the tongue, which includes the glands of Blandin-Nuhn and von Ebner, the buccal, labial, hard and soft palates, the palatoglossal glands and a limited number in the anterior part of the floor of the mouth (1, 5). The saliva produced by the submandibular gland is considered the basal salivary flow at rest (6). On stimulation, the parotid secretion rises to 50%.

Within the salivary glands, saliva is produced by the acinar cells that can be classified as either serous or mucous. The composition of the saliva excreted by the individual glands show considerable variation. For example, the parotid glands and the von Ebner's glands on the back of the tongue contain exclusively serous acini and therefore produce serous (watery) saliva. In contrast, the sublingual glands and most of the minor salivary glands are mixed mucoserous with a predominance of mucous acini and secrete saliva rich in mucins which resulting in the production of visco-elastic saliva, mainly due to the presence of MUC5B *i.e.* a large, water-binding glycoprotein (7). The submandibular gland acini produce seromucous saliva (5,6,8).

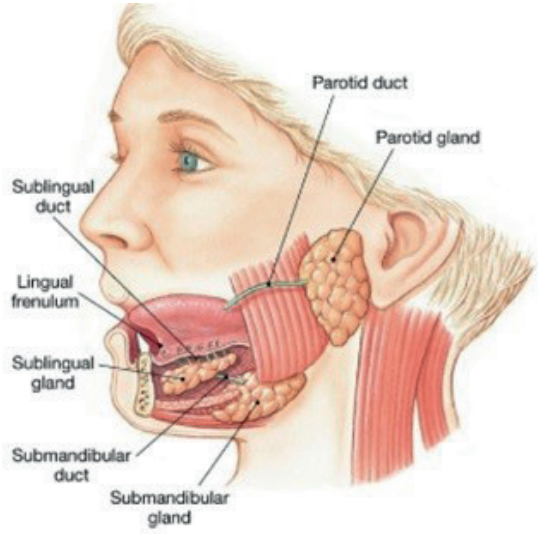

Figure 2 - The location of the three major salivary glands and their ducts (8).

Anatomically, salivary glands consist of lobes with different compartments: secretory acini, intercalated ducts, striated ducts, and excretory ducts. Contraction of the myoepithelial

cells, which overlay the glands, support the transport of saliva to the orifices in the floor of the mouth and in the inside of the cheeks. The excretory duct of the submandibular gland is called the submandibular or Wharton's duct and of the parotid gland is called the parotid or Stensen's duct (Figure 2). Both excretory ducts are about 5 cm long. An anatomical difference is that Stensen's duct mainly runs horizontally while Whartons duct, after an initial horizontal section, bends upwards to the floor of the mouth. The sublingual gland has 8-20 secretory ducts (ducts of Rivini) and sometimes a major duct (Bartholin's duct) that may either drain in Whartons duct or in the sublingual caruncle. Minor salivary glands have their own single excretory duct which opens into the oral cavity (3, 6, 9).

Considering the many protective functions of saliva, it is important that a sufficient amount of saliva is continuously secreted into the oral cavity. The salivary glands and their excretory ducts can, however, be affected by a wide range of conditions that result in a temporary or permanent reduction of saliva secretion. As age increases, there is a reduction in the number of functional secretory cells with an increase of adipose and fibrous tissue (10). In non-medicated older persons there seems to be a slight decreased resting flow rate and a more or less healthy stimulated flow rate (11). Autoimmune diseases such as Sjögren's syndrome and cystic fibrosis, sarcoidosis and radiotherapy for head and neck tumors may result in an irreversible reduced flow rate under resting, as well as stimulated conditions due to destruction and atrophy of the acini. Also, the use of multiple xerogenic medications (polypharmacy), such as antidepressants, anxiolytics, opiates, antihypertensives, diuretics and antihistamines, have significant, long-term negative side-effect on saliva secretion rates while exposure to stressful situations may give a short-term inhibition of saliva secretion (9). Polypharmacy may cause xerostomia or hyposalivation, where each drug itself does not have to induce these conditions. In all these circumstances, there is reduced secretion of saliva by the secretory cells in the glands. There are also situations where the production of saliva by the gland is normal, but the transport of the produced saliva to the oral cavity is impaired. This is the case when the excretory or intraglandular duct(s) are blocked whereby the obstruction can be caused by narrowing of the duct, a mucous plug or a salivary stone (12).

Salivary stones

Salivary stones (sialoliths) are calcified structures or concretions. Salivary stones are a relatively rare phenomenon with an estimated prevalence of 1 in 10.000-30.000 (13, 14). They usually affect the submandibular main duct and intraglandular ducts in 72 to 95%

of the cases, whereas the parotid main ducts and intraglandular ducts are affected in 4 to 28% of the patients. Salivary stones in the sublingual and minor salivary glands are very rare, and comprise only 0.4 to 7% of all cases (13, 15-18). Single salivary stones occur in 70-80% of the cases and multiple stones are more often found in the parotid ductal system. Most of the sialolithiasis occur in the fourth and fifth decades of life (19), and there is no gender predilection (13, 20).

Salivary stones are most commonly found in the submandibular system due to its anatomic nature and salivary viscosity. The main duct is located superior to the position of the gland and saliva has to be transported against the force of gravity. Also, Wharton's duct is relatively long and it has two more or less acute bends, one at the posterior border of the mylohyoid muscle and one directly for the sublingual papilla. Submandibular salivary viscosity is higher than that of parotid saliva and unstimulated saliva has a higher viscosity than stimulated saliva (21, 22).

Aetiology

The aetiology of salivary stones is still not completely understood, and various hypotheses have been put forward. These hypotheses include the agglomeration of sialomicroliths (19), anatomical variations of the salivary ducts (23) and an altered biochemical composition of saliva (12, 24). A decreased salivary flow rate or salivary stasis could also contribute to the development of salivary stones by increasing the risk of calcium salts precipitation.

Clinical features and diagnosis

Characteristic clinical symptoms of sialolithiasis are mealtime associated pain and swelling of the affected salivary gland. If a salivary stone obstructs the salivary duct, this will lead to accumulation of saliva in the gland and increased intraglandular pressure, resulting in pain and swelling, especially upon stimulation of salivary flow, *e.g.* during eating. Afterwards, symptoms will gradually disappear because the obstruction of the excretory duct is rarely absolute, which enables a gradual discharge of the accumulated saliva (17).

The volume of salivary stones increases gradually over time. The exact grow rate is not known but the average radial increase is estimated at 0.5 mm per year (25). Due to the increased volume of the salivary stone, the associated symptoms will appear more quickly and disappear more slowly. Prolonged presence of a salivary stone increases the risk of

inflammation, atrophy and fibrosis of the affected salivary gland. In 90% of the patients with a salivary stone, infection of the affected gland is present and in 12-18% a purulent discharge may be observed (17, 19, 26).

Sialolithiasis may be asymptomatically, and incidentally found during routine dental radiographic examination. Yet, most patients with sialolithiasis will present in the clinic during an acute phase of the disease, and may complain about unilateral (radiating) pain and swelling. Sometimes sialolithiasis cause a painless swelling due to a chronic sialadenitis or a large stone. Anamnesis and physical examination with bimanual palpation of the floor of the mouth or palpation around Stensen's duct is mandatory. By gently pressure on the gland the quality, quantity and clinical aspect of the saliva is evaluated at the orifice (27). An acute suppurative sialadenitis may occur in the presence or absence of a stone and can cause, swelling, pain, malaise, headache and trismus and by massage of the gland sometimes purulent saliva or even frank pus can be observed. Additional imaging studies are necessary to detect a salivary stone as a possible cause for the disease. Different imaging modalities are possible and each of these techniques has its own advantages and disadvantages with regard to the use of ionizing radiation, costs, availability and the ability to visualize the ductal system. The possible techniques include: x-rays (solo recordings and panoramic radiographs), medical computed tomography (CT), cone beam (CB)-CT, magnetic resonance imaging (MRI), ultrasound and sialography. In daily practice, CBCT and ultrasound are mainly used (28-30). 80-95% of the submandibular and 40-60% of the parotid stones are radiopaque (15, 27). When there is a strong indication of obstruction and/or a stone on clinical grounds, but nothing is visible radiologically, diagnostic sialendoscopy may be used to visualize the salivary duct system and the possible cause for the obstruction. Sialendoscopy was introduced about 20 years ago and revolutionized the diagnosis and management of obstructions. It is increasingly used when the patient's symptoms are indicative of a possible salivary stone, mucous plug or salivary duct stricture or stenosis (30-32).

Treatment

When a symptomatic salivary stone has been identified, treatment is aimed at removal of the stone, preservation of the affected gland, restore normal saliva production and recovery of the inflammation. Preferably, a non-invasive procedure is applied, with the least possible discomfort and risk of complications for the patient. Decisive parameters when choosing the most appropriate treatment method are the size of the salivary

stone, its location (whether it is located in the excretory duct, distal duct, hilar region, or intraparenchymal ductal system), number of stones present and the interaction of the stones with the surrounding tissue (adhesive, impacted, mobile) (33, 34).

The non-invasive treatment options range from prescribing an acidic diet and the use of sialogogues to massage of the affected gland. All these methods are aimed at stimulating the salivary secretion, with the idea that a stone with a small diameter is flushed away. When these conservative interventions do not have the intended effect, and the stone is positioned near the orifice or in the excretory ductus up to the hilar region, the stone can be removed surgically via an intraoral approach, under local anesthetics (35, 36). Impacted stones or stones with a diameter exceeding 5mm, located in the duct up to the first order branch which are not retrievable by the aforementioned treatment options might be exposed to extracorporeal shock-wave lithotripsy. The applied vibrating shock waves will fragment the salivary stone into smaller fragments that are either flushed away or could be removed by an intraoral approach (37).

Another non-invasive approach for stone removal is sialendoscopy. An endoscope with a very small diameter (0.6mm) is introduced into the duct after the orifice has been dilated. Sialendoscopy is performed while the patient is under local or general anaesthesia. During the endoscopy, irrigation is performed continuously with 0.9% saline, a local anaesthetic solution and sometimes an anti-inflammatory rinse. This dilates the lumen of the ducts and provides anaesthesia and cleansing (34). Diagnostic sialendoscopy is performed with a single channel device, while interventional sialendoscopy is performed with a double channel device, allowing the use of an expandable wired basket to capture the salivary stone. Sialoliths, smaller than five millimeter in section and mobile stones located in the main excretory duct as far as the first- and second-order branches, can be extracted endoscopically from both the submandibular and parotid gland (33, 34, 38). Removal of sialoliths from the intraglandular part of the parotid gland, may be addressed through a combined endoscopic and incisional approach technique. The success rates of sialolith treatment are high (>90%). Recurrence of sialolithiasis is rather uncommon, and is estimated to occur in 1–10% of the patients (36, 39, 40). In some cases, when the aforementioned non-invasive methods fail and the patient continues to have symptoms, the affected gland has to be surgically removed.

Size and structure

Salivary stones are calcified solid structures with a yellow or yellowish-brown color, and vary greatly in size and weight (Figure 3). Generally, submandibular stones are larger than parotid stones (15). Approximately 60% of the stones have a diameter between 2.1-10,0 mm and 8% are larger than 15 mm in section. These larger salivary stones are known as 'giant salivary gland calculi', mainly affect middle-aged male patients and usually originate in the submandibular gland (16, 41).

The shape of the salivary stone and its surface structure are related to the location where the stone develops. Stones originating from the extra-glandular ductal system are usually elongated and smooth, whereas stones originating from the hilus or intraglandular part ductal system are round or oval and have a relatively rough surface (27).

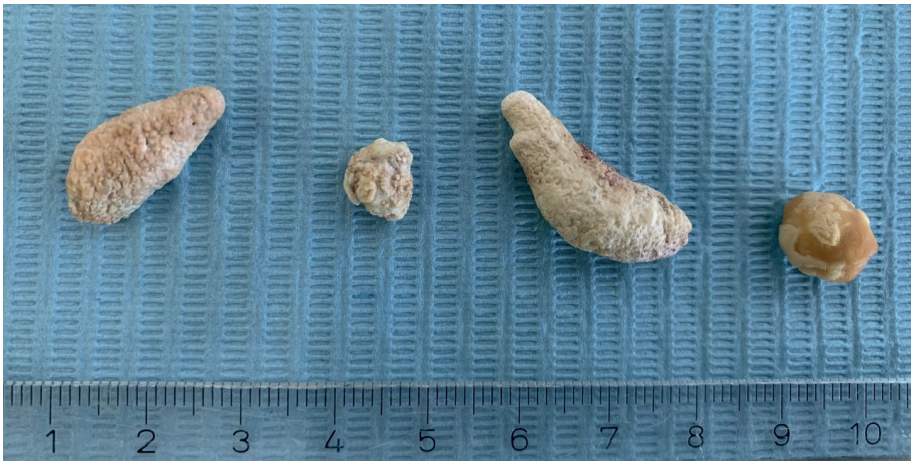

Figure 3 – These examples illustrate the wide variation in color, size, shape and surface structure of submandibular salivary stones.

Sialoliths are composed of a central nucleus from mainly organic matter, surrounded by concentric or irregular inorganic layers, with varying degrees of mineralization. Sialoliths are comprised primarily of inorganic material whereby submandibular stones contain between 70–80% of inorganic material and parotid stones approximately 50%. The majority of inorganic components are mixtures of calcium and phosphates in the form of hydroxyapatite, (amorphous) carbonated calcium phosphate, whitlockite and brushite. When infection is present, ammonium and magnesium can also be present in salivary

stones. The organic material of salivary stones, mainly found in the nucleus and on the outer layers, comprises proteins, lipids and carbohydrates. The relative contribution of organic material in salivary stones varies greatly and varies in different parts of sialoliths between 23-100% (12, 42, 43).

Aim of the thesis

The aim of this thesis was to gain detailed insight into the biochemical composition and formation of salivary stones. For this, an overview of the recent scientific literature on salivary stones is given, with regard to symptoms, etiology, biochemical composition and treatment options in **chapter 2**. **Chapter 3** presents a study on imaging of salivary stones and describes the relationship between the volume of submandibular salivary stones *in vivo* determined with CBCT and the post-operative volume *in vitro* with micro-CT.

The kidneys and gall bladder can, similarly like the salivary glands, be obstructed by kidney stones and gall bladder stones, respectively. As it has been reported that the risk of these stones might be related to specific morbidities (44, 45), we performed a case control study on the possible relation between systemic diseases and the risk of developing a salivary stone (**chapter 4**). Research on the inorganic biochemical composition of salivary stones in relation to stone- and patient-related factors is presented in **chapter 5**, while research on the protein composition of submandibular salivary stones is presented in **chapter 6**. Finally, **chapter 7** attempts to bring this thesis to a contemplative conclusion.

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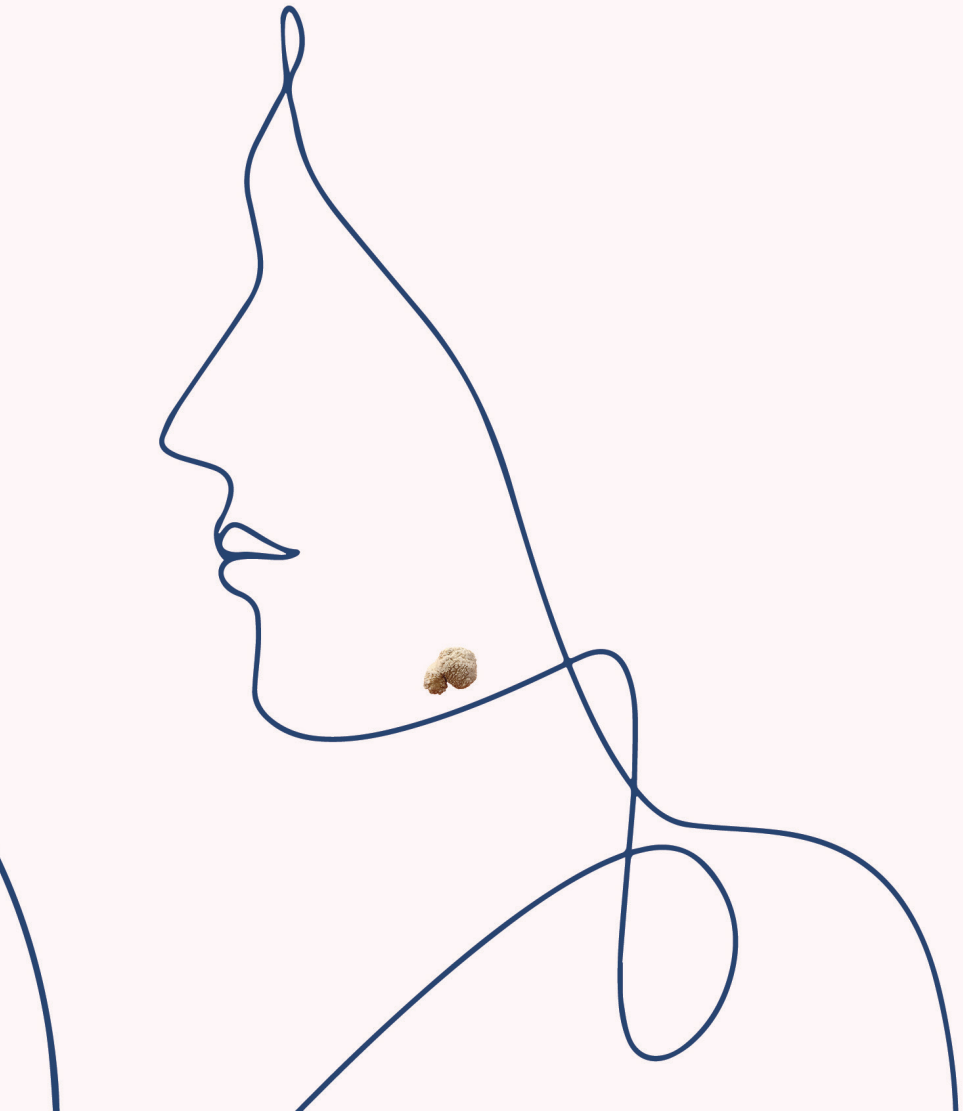

CHAPTER 2

Salivary stones: symptoms, aetiology, biochemical composition and treatment

S Kraaij
KH Karagozoglu
T Forouzanfar
ECI Veerman
HS Brand

British Dental Journal 2014 Dec 5;217(11):E23

ABSTRACT

Salivary stones, also known as sialoliths, are calcified concrements in the salivary glands. Sialoliths are more frequently located in the submandibular gland (84%), than in the parotid gland (13%). The majority of the submandibular stones are located in Wharton's duct (90%), whereas parotid stones are more often located in the gland itself.

Salivary stones consist of an amorphous mineralized nucleus, surrounded by concentric laminated layers of organic and inorganic substances. The organic components of salivary stones include collagen, glycoproteins, amino acids and carbohydrates. The major inorganic components are hydroxyapatite, carbonate apatite, whitlockite and brushite.

The management of salivary stones is focussed on removing the salivary stones and preservation of salivary gland function which depends on the size and location of the stone. Conservative management of salivary stones consists of salivary gland massage and the use of sialogogues. Other therapeutic options include removal of the stone or in some cases surgical removal of the whole salivary gland.

INTRODUCTION

Salivary stones or sialoliths are calcified structures or concretions located in the parenchyma or ductal system of the salivary glands. (Figure 1). Sialolithiasis is a common salivary gland disorder characterized by the obstruction of the salivary secretion, accounting approximately one third of the salivary gland disorders.[1]

Figure 1 - Fragmented sialolith, removed from the submandibular gland

Most cases of sialolithiasis present with symptoms such as pain and swelling in the submandibular or parotid region during mealtime (Table 1). Swelling is the most common symptom in submandibular stones, followed by pain. Three percent of the patients with a submandibular stone have no symptoms. Swelling is also the most common symptom of a parotid gland stone. Pain is present in approximately a half of the patients with a parotid stone and only 1% has no symptoms.[2,3,4] In general, pain and swelling are more pronounced when a stone is located in the duct than when the stone is located in the gland itself.[2]

	Submandibular stone	**Parotid Stone**
Swelling	86 - 92 %	88 – 93 %
Pain	35 – 47 %	47 – 55 %
No symptoms	3 %	1 %

Table 1 - Frequency of swelling and pain in patients with salivary stones

Characteristic for sialolithiasis are episodes of pain and swelling during mealtime which may persist a few hours, followed by long episodes of remission (weeks or months).[2,5] The pain and swelling are caused by the obstruction of the salivary flow in the affected gland, resulting in accumulation of saliva and a subsequent increase in intraglandular pressure. In incomplete obstruction of the duct, saliva can seep through or around the sialolith. In these cases, a salivary stone can be symptomless and these stones may be an incidental finding on a dental panoramic radiograph.[6]

The duration of symptoms before patients present in a clinic varies considerably. The mean duration of symptoms is approximately five years and four months for submandibular stones and four years and ten months for parotid stones.[7] One third of the patients with sialolithiasis will present within the first six months of symptoms.[2]

In 90% of the patients with a salivary stone, infection of the affected gland is present and in 12-18% a purulent discharge is seen.[2,8,9]

The salivary flow rate of patients with sialolithiasis decreases when the mass of the sialolith increases. The mean submandibular salivary flow rate in patients with a submandibular stone was 38% lower compared to healthy individuals (0.18 and 0.29 ml/min, respectively).[10] Nishi et al[11] observed a mean reduction of 84% of the salivary flow rate of the affected submandibular gland compared to the unaffected contralateral gland. Long-term obstruction of the salivary flow may increase intraglandular pressure, leading to destruction of the salivary gland and formation of connective tissue. Dissected submandibular salivary glands showed periductal and interlobular fibrosis, lymphocytic infiltration and atrophy of the acinar cells.[12,13,14]

Salivary stones usually have a yellow or yellow-brown colour, and vary greatly in size and weight. The weight of salivary stones varies from 1 mg to almost 6 g, with an average weight of 300 mg.[15] Generally, submandibular stones are slightly larger than parotid stones (Table 2). [3,4,5,16] Approximately 59% of the stones have a diameter between 2.1 and 10mm and 7.6% are larger than 15mm in section.[17] Salivary stones are classified as 'giant salivary stones' when the diameter is 15 mm or more in any direction or when the weight is 1gram or more. Giant salivary stones are usually located in the glandular parenchyma and are rarely found in Wharton's or Stenson's ducts.[18]

The shape of a salivary stone depends on the location from where the stone originates. Stones originating from the ductal system are mostly elongated, whereas stones

originating from the hilus or gland are round or oval.[1,2,17,19] Generally, submandibular stones have a smoother surface whereas parotid stones have a more irregular surface.[3]

	Submandibular stone	Parotid Stone
Incidence	72 - 95 %	4 - 28 %
Average size	8.3mm (2 - 30)	6.4mm (4 - 15)
Average age	43 years (40.5 - 48)	49.8 years (47.8 - 52.6)

Table 2 - Characteristics of submandibular and parotid sialoliths

Incidence and distribution between glands

The mean incidence of hospital admission for patients with symptomatic sialolithiasis in the United Kingdom is 27.5 per million per year, and the estimated prevalence of sialolithiasis is 0.45% in an average life expectancy of 76 years.[16] This percentage is considerable lower than the previously frequently reported 1.2% based on post-mortem research by Rauch and co-workers.[20]

Sialolithiasis is most common in the fourth and fifth decade of life. The average age of patients with submandibular stones is slightly younger than that of patients with parotid stones (Table 2).[2,3,4,21] Sialolithiasis in minor salivary glands seems to occur later in life, in the fifth to eight decades. Sialolithiasis in the first decade of life is rare, and encompassing 2.9% of all cases.[17] Sialolithiasis is familial in approximately 1% of the cases.[3] Bullock et al[22] reported on a family in which three successive generations (grandmother, mother and grandchild) had salivary stones in both submandibular and parotid glands.

Until the nineties of the last century, most studies reported a male predominance of sialolithiasis, varying from 2.5:1 to 1.2:1.[2,8,17,23,24] However, more recent studies reported an almost equally distribution of salivary stones between men and women.[16,25]

Salivary stones are equally distributed between the left and right side of the oral cavity.[2,5,17,26,27] In 70-80% of the patients a single stone is found, in 20% two salivary stones are found and in roughly 5% three or more salivary stones are found in the affected salivary gland.[17]

The submandibular gland is affected in 72 to 95% of the cases, whereas the parotid gland is only affected in 4 to 28 % of the patients. Salivary stones in the sublingual and minor salivary glands are rare, and comprises only 0.4 to 7% of all cases.[2,3,5,17,20,25]

Submandibular stones are usually located in the duct (80 - 90%), of which 57% is located in the hilum and 34% is located in the distal duct. Ten per cent of the submandibular stones is located in the gland itself.[3,28] Twenty-three per cent of the parotid stones is located in the parenchymal system, 13% is located in the hilum and 64% is located in the distal duct.[3]

Sialoliths of the minor salivary glands are most frequently located in the upper lip (47%), the buccal mucosa (35%) and lower lip (10%). Only a few case reports described sialolithiasis of the minor salivary glands of the tongue and palate.[1,12,21,29,30,31]

Etiology

The exact etiology of salivary stones is not completely understood, and various hypotheses have been put forward. These hypotheses including the agglomeration of sialomicroliths, anatomical variations of the salivary ducts and an altered biochemical composition of saliva. It is considered that salivary stasis or decreased salivary flow contributes to the precipitation of calcium.[14]

Sialomicroliths

A sialomicrolith is a microscopic concretion in a salivary gland. These concretions consist of crystals containing calcium and phosphorus, as well as organic secretory material in granular form and necrotic cell residues.[28,32] Sialomicroliths have been identified in serous acinar cells, striated ductal cells, lumen and interstitium of almost all normal submandibular glands and in 10 to 20% of the normal parotid glands.[21,33,34,35,36,37] The observation that sialomicroliths were more frequently found in the submandibular gland may correspond to a higher calcium concentration in submandibular glands.[36] The size of microcalculi differs per place of origin; intracellularly sialomicroliths can be up to 25µm, in acinar lumen up to 70µm and interstitially up to 35µm.[14,33]

The incidence of sialomicroliths is related to the age of the patient, with an increased sialomicrolith formation in the intraglandular duct system of the submandibular gland in patients of 40 years or older.[28,34] Secretory inactivity of a normal salivary gland also leads to increased formation of sialomicroliths.[36] Sialomicroliths usually form in autophagosomes in normal salivary glands, enter the lumen and pass from the salivary gland in the saliva unnoticed. Occasionally, the sialomicroliths might become impacted

resulting in local micro-obstruction. This micro-obstruction may cause atrophic foci and chronic sialendenitis.[1,33,34,38] These micro-obstructions may clump together into a salivary stone. However, the incidence of sialomicroliths is not related to the duration of symptoms of sialolithiasis.

Microorganisms

Microorganisms do not seem to play a significant role in the initiation of salivary stones, as very few studies identified microorganisms in the nuclei of sialoliths.[31,39,40,41,42] However, in the intermediate and peripheral parts of sialoliths microorganisms have been identified, mostly oral commensal bacteria like *Streptococcus* or *Peptostreptococcus* species.[14,29,39,43,44,45,46] The external surfaces of calculi removed from infected salivary glands, were found to be covered with a heavy accumulation of filamentous and rod shaped bacteria with a diameter of 0,5 – 1 µm.[28,40,44]

Salivary gland anatomy

The anatomical differences between Wharton's and Stenson's duct may favour the formation of sialoliths in the submandibular gland. Although the diameter of both ducts is comparable, Wharton's duct is longer and has a bow-shaped course in the cranial direction.[21,47] This results in a flow against gravity, which may facilitate stasis of submandibular saliva.[23,25,35] Furthermore, submandibular gland saliva is more viscous than parotid gland saliva, due to a higher mucin concentration. Additionally, saliva from the submandibular gland has a higher pH, and contains twice as much calcium as parotid saliva.[1,28,48,49] Mineralization is supported by accumulation of calcium and an increase in pH, which decreases the solubility of calciumphosphate in saliva. Together, these factors may favor mineralization of a mucoid gel formed in the ductal system of a submandibular gland.[28,50]

Predisposing factors

An altered saliva composition may predispose to the formation of salivary stones. It has been reported that the salivary protein content and viscosity of saliva is higher in patients with sialolithiasis than in unaffected individuals.[51] Several studies showed higher calcium concentrations in saliva in patients with salivary stones than in a healthy control group.[10,21,36,52] Saliva of patients with salivary stones also contains reduced concentrations

of the crystallisation inhibitors phytate, magnesium and citrate, which may predispose to the formation of salivary stones.[10,42] Salivary concentrations of sodium, chloride, nitrate, phosphate and sulphate did not differ from healthy individuals.

Systemic factors

Twenty-six percent of the patients with salivary stones have one or more comorbidities.[3] Several studies suggested that patients with salivary stones suffer more frequently from kidney stones or stones in the gallbladder.[17,21] However, in other studies the incidence of confirmed cholelithiasis and nephrolithiasis did not differ from the general population.[3,25] The high prevalence of diabetes and hypertension in an Italian population with salivary stones did not correspond to other studies.[3,17,53] Gout appears to be the only systemic disease predisposing to sialolith formation.[1] Salivary stones in gout patients are predominantly composed of uric acid.[19]

A decreased salivary flow rate may facilitate the formation of a salivary stone. However, there is no increased incidence of sialolithiasis in patients suffering from Sjögren's syndrome.[42] The use of diuretics predisposes to formation of salivary stones, because diuretics decrease the salivary flow rate.[54] 20 percent of the patients with salivary stones use diuretics, twice that of patients without salivary stones.[25]

Smoking may decrease the antimicrobial activity of saliva, resulting in an increased bacterial load and inflammation of the salivary duct and/or gland. Huoh et al[25] found a higher rate of smoking or history of smoking in patients with salivary stones than in the general population, although the difference did not reach statistical significance. Submandibular stones of smokers are also slightly larger (average diameter 8.7 mm) than submandibular stones of non-smokers (7.9mm). On the other hand, the mean age of presentation of a salivary stone is higher in smokers (50.9 years) than in non-smokers (44.9 years).[25]

It has been suggested that residents of hard water areas are at increased risk of developing salivary calculi. However, there is no correlation between the incidence of salivary stones and the calcium content of water in different regions in England.[55]

Structure

Submandibular and parotid sialoliths consist of an amorphous, mineralized core or nucleus, with concentric laminated layers or shells of organic and inorganic

substances.[21,23,28,41,44,49,50,56,57,58,59] (Figure 2). The nucleus of the sialolith has a diameter between 0.5 and 1.5 mm and a softer consistence than the peripheral parts.[19,28] The core is usually homogeneous but may contain substructures, indicating fusion of smaller structures like sialomicroliths.[21,29] The concentric laminated layers are either homogenous or extremely irregular, suggesting that sialolith formation is an intermittent process.[23,50] Scanning electron microscopy showed granular or globular, coarse structures with a diameter of 5 - 15µm and pyramid structures on the surface of submandibular calculi.[40,49,58] Hexagonal, needle-like and plate-shaped crystals on the surface of sialoliths have also been described.[46]

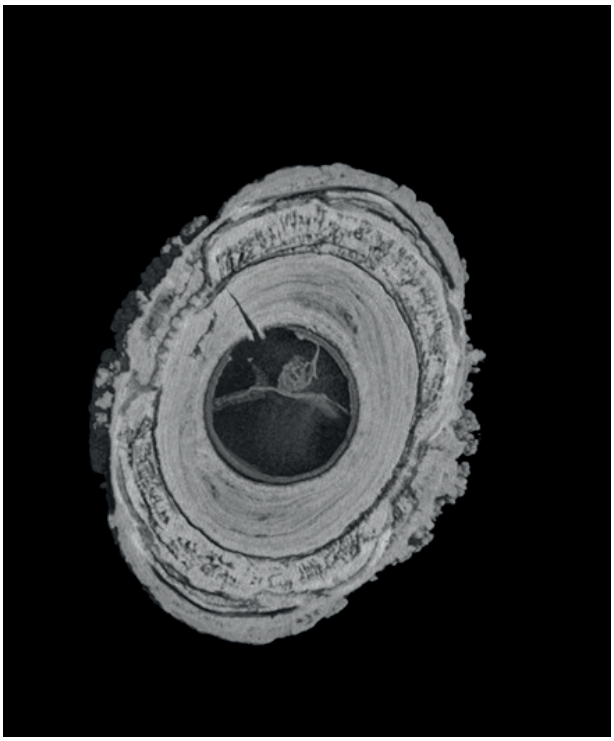

Figure 2 - Micro CT scan of a sialolith, showing an organic nucleus surrounded by laminated layers of organic and inorganic substances (courtesy of professor Johan Aps, University of Washington, School of Dentistry)

Biochemical composition

Sialoliths consist of both organic and inorganic material, but there is a great variation in the relative contribution.[42,60] The organic matrix varies in different parts of sialoliths from

23 to 100% and is mainly present in the nucleus of the sialolith and the other shell of the sialolith.[25,28,36] Few sialoliths are constituted of only organic material; most salivary stones contain calcium phosphates, either as hydroxyapatite or carbonate apatite.[52,61]

The organic matrix comprises approximately 20 percent of the dry weight of parotid stones. Submandibular stones contain less organic material: approximately 9 to12 per cent of the dry weight.[62,63] The organic part consists of collagen, neutral and acid glycoproteins, other proteins, lipids and carbohydrates such as glucose and mannose.[33,60,61,63] In submandibular stones, protein contributes approximately 5% to the stone weight whereas lipids contribute 1%.[61,63]

Proteins

In submandibular stones, protein contributes approximately 5% to the stone weight. The matrix of human submandibular stones contains higher levels of proteins than the matrix of parotid stones.[63] Using immunoblotting techniques, Proctor and co-workers[64] observed a large, unidentified glycoprotein in solubilised submandibular sialoliths. Lower molecular weight proteins, including statherin and acidic proline-rich protein, were also present in stones. These calcium-binding proteins are present in human submandibular saliva, and probably bind to calcium ions in sialoliths.

Amino acid analysis of the proteins in submandibular sialoliths showed relatively high levels of alanine, leucine, glutamine, aspartic acid, valine and glycine. Lysine, arginine, proline, methionine, cysteine, histidine, serine, isoleucine, phenylalanine, tyrosine and threonine were present in lower amounts.[15,62]

Lipids

Lipids are present in the organic matrix of both submandibular and parotid stones.[65] The lipid content is not related to the weight of the stone.[66] In parotid salivary stones, these lipids mainly comprise neutral lipids (74%), glycolipids (17%) and phospholipids (9%). Of the neutral lipids, about 77 per cent are present as free fatty acids, 14 per cent as cholesterol and small percentages as triglycerides and cholesterol esters.[61] The phospholipid composition of sialoliths is comparable to that of plasma membranes and does not resemble lipids in saliva or bacterial membranes.[66] Slomiany and co-workers[61,63] suggested that the lipids and phospholipids are important for the initiation of the mineralization of a salivary stone.

Inorganic matrix

Sialoliths are mainly composed of inorganic material. Submandibular stones contain between 70 and 80% and parotid stones around 50%.[15,42,66,67]

The mineral component is proportional to the size of the sialolith, suggesting that mineralization of the organic matrix increases with time.[36,42]

Hydroxyapatite ($Ca_5(PO_4)_3OH$) is present in all submandibular stones, frequently together with whitlockite ($Ca_3(PO_4)_2$). Octacalciumphosphate ($Ca_8H_2(PO_4)_6.5H_2O0$ and brushite ($CaHPO_4.2H_2O$) are less often identified. Whitlockite is especially found in sialoliths from Wharton's duct and often present in the nucleus. Parotid salivary stones also contain always hydroxyapatite. Whitlockite and octacalcium phosphate are more frequently present in parotid stones than in submandibular stones.[28,45,50,64,68,69]

Minor inorganic components of sialoliths are potassium, sodium, ferrum, silicon, brimstone and chloride.[10,70] Stones retrieved from an infected gland may also contain, ammonium and magnesium.[52]

Diagnosis

Most patients suspected of sialolithiasis will present in the clinic during an acute phase of the disease. A careful anamnesis and physical examination of the patient are important. The physical examination should include bimanual palpation of the floor of the mouth in a posterior to anterior direction for submandibular glands or an intraoral palpation around Stensen's duct for parotid glands.[1,31,48,71] The affected gland may feel firm and tender. In case of a submandibular gland, the affected side of the floor of the mouth may be elevated and inflamed.[26]

In addition to an oral examination, several imaging techniques can be applied. Despite the relatively high percentage of inorganic material in salivary stones, between 80 and 95 percent of the submandibular stones and 43 to 60 percent of the parotid stones are radiopaque.[1,5,17,31] Demonstration of sialoliths by radiographic examination is effective in approximately 80% of the cases.[72] (Figure 3).

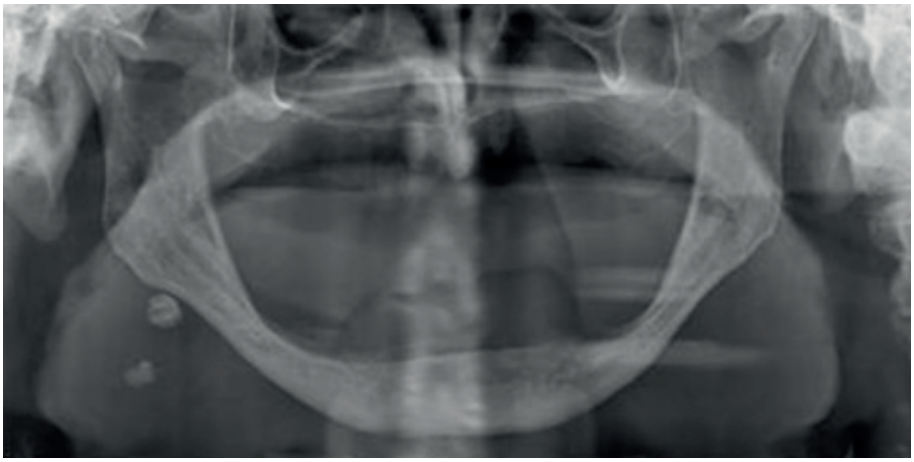

Figure 3 - Panoramic radiograph of an edentulous patient with sialoliths in the right submandibular gland (courtesy of professor Johan Aps, University of Washington, School of Dentistry)

The detection rates differ between intraoral occlusal radiographs and extra-oral panoramic radiographs. Extra-oral radiographs will detect fewer salivary stones because many calculi will be projected superimposed on bony structures or teeth.[73] This indicates that an occlusal radiograph is the most useful method for detection of a submandibular sialolith.[74] Computer tomography (CT) and cone beam computer tomography (CB-CT) can detect any size of sialolith, but have the disadvantage of a relatively high radiation dose.[73] Ultrasonography allows detection of stones with a diameter of 2 mm or more.[1,4,73] This technique has the additional advantage that it also can be used during an acute episode of sialendenitis.

Radiolucent sialoliths can be imaged with sialography. A contrast agent is injected into the duct of the affected gland and subsequently radiographs are taken. Sialography is most frequently used for detection of parotid sialoliths. It is contraindicated during acute episodes of sialendenitis and in patients with an allergy to contrast media. Sialograms are up to 100% effective in detecting intraductal and intraglandular calculi.[1,75]

Sialendoscopy is a minimally invasive technique to visualize the salivary duct system, usually performed under general anaesthesia. An endoscope with a very small diameter (0.6 mm) is introduced into the duct after the orificium has been dilated with special instruments with increasing diameters from 0.8 to 1.6mm. (Figure 4 and 5) The endoscope has a rinse channel that can be used to flush the duct with saline or an anti-inflammatory

rinse. This flushing primarily results in a better image of the salivary ducts but may also have a therapeutic effect.76,77

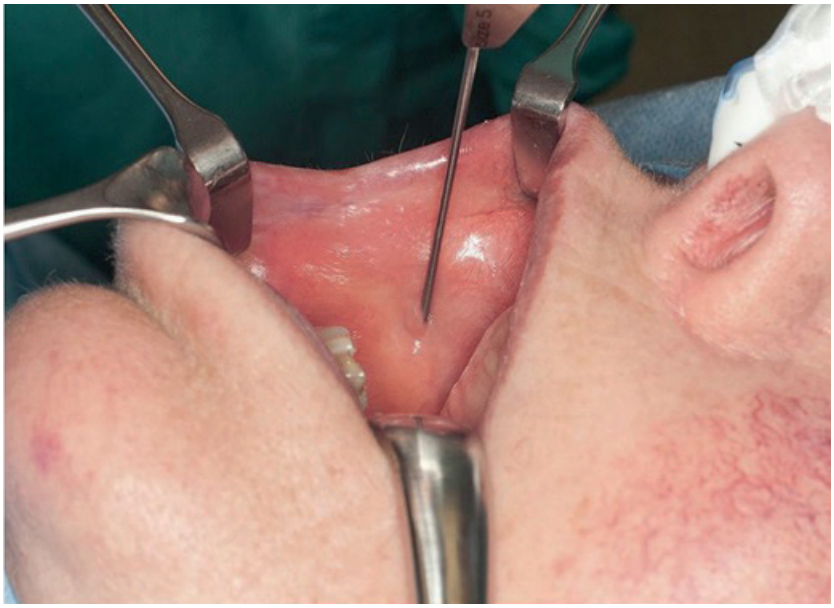

Figure 4 - The oroficium of the parotid gland is dilated and prepared for sialendoscopy with a special probe (courtesy of dr. Erik van der Meij, department of Oral-Maxillofacial Surgery, Medical Centre Leeuwarden)

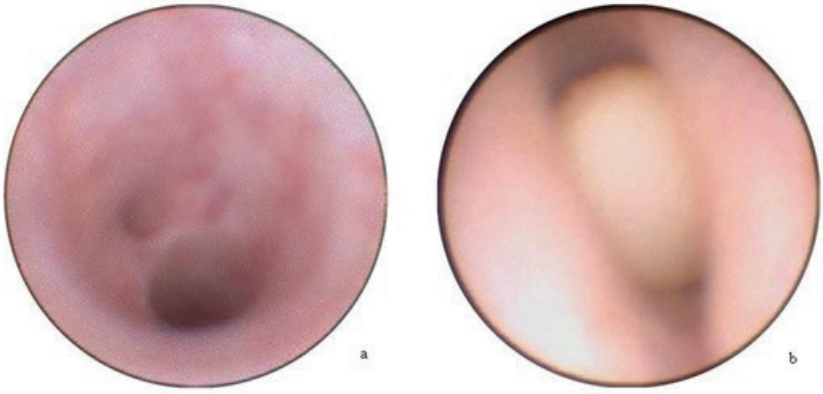

Figure 5 - Sialendoscopic images of the bifurcation of Stenson's duct (a) and a sialolith in Stenson's duct (b) (courtesy of dr. Erik van der Meij, department of Oral-Maxillofacial Surgery, Medical Centre Leeuwarden)

Treatment

The primary objective in the treatment of sialolithiasis should be preservation of gland function in combination with a low level of complications and discomfort for the patient.[78] Non-invasive conservative management of sialolithiasis consists of gland massage, in combination with use of sialogogues and irrigation. This treatment has the highest success rate when stones are small and located in the duct. When an infection is suspected, antibiotics should be prescribed.[1,31,48]

Nearly all intraductal submandibular and parotid stones can be removed by a relatively simple intraoral approach under local anaesthesia.[5,31] (Figure 6). This includes submandibular stones located near the knee of the duct.[24] Transoral removal is treatment of choice in patients with stones that can be palpated bimanually and/or which are localized by ultrasound within the prehilar region of the gland.[78,79] After stone removal, it is recommended that salivary gland massage is carried out several times a day, combined with a sour diet and sialogogues to stimulate the salivary flow.[79] It is recommended to avoid use of sutures in the incised duct as this may increase the risk of scarring.[71,73,75]

Invasive management of sialolithiasis may consist of extracorporeal shock-wave lithotripsy, sialoendoscopy or surgical removal.

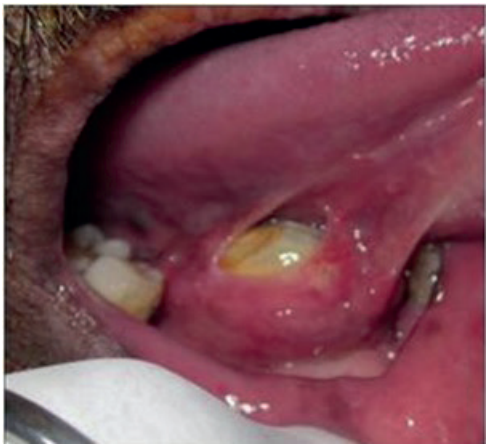

Figure 6 - Transoral removal of a submandibular sialolith after a surgical incision of 5 mm has been made in the oroficium of Wharton's duct (courtesy of professor Johan Aps, University of Washington, School of Dentistry)

Most patients experience no complaints or discomfort, but a normal functioning gland, after sialolith therapy that does not include surgical removal of the gland.[80,81,82]

After transoral surgical removal of submandibular stones, the secretion rate of the treated gland is in 75% of the cases similar to that of the contralateral gland.[24] Recovery of function of the salivary gland is related to factors such as glandular infection, the diameter of the sialolith and the age of the patients. Patients with normal salivary secretion from a submandibular gland after transoral removal of a sialolith were significantly younger than those patients in which saliva secretion from the treated gland was decreased compared to the contralateral unaffected submandibular gland. The duration of symptoms before treatment was not related to recovery.[11,24,83]

Recurrence of sialoliths is rather uncommon, and is estimated to occur in 1 to 10% of the patients.[17,72,78,79,84]

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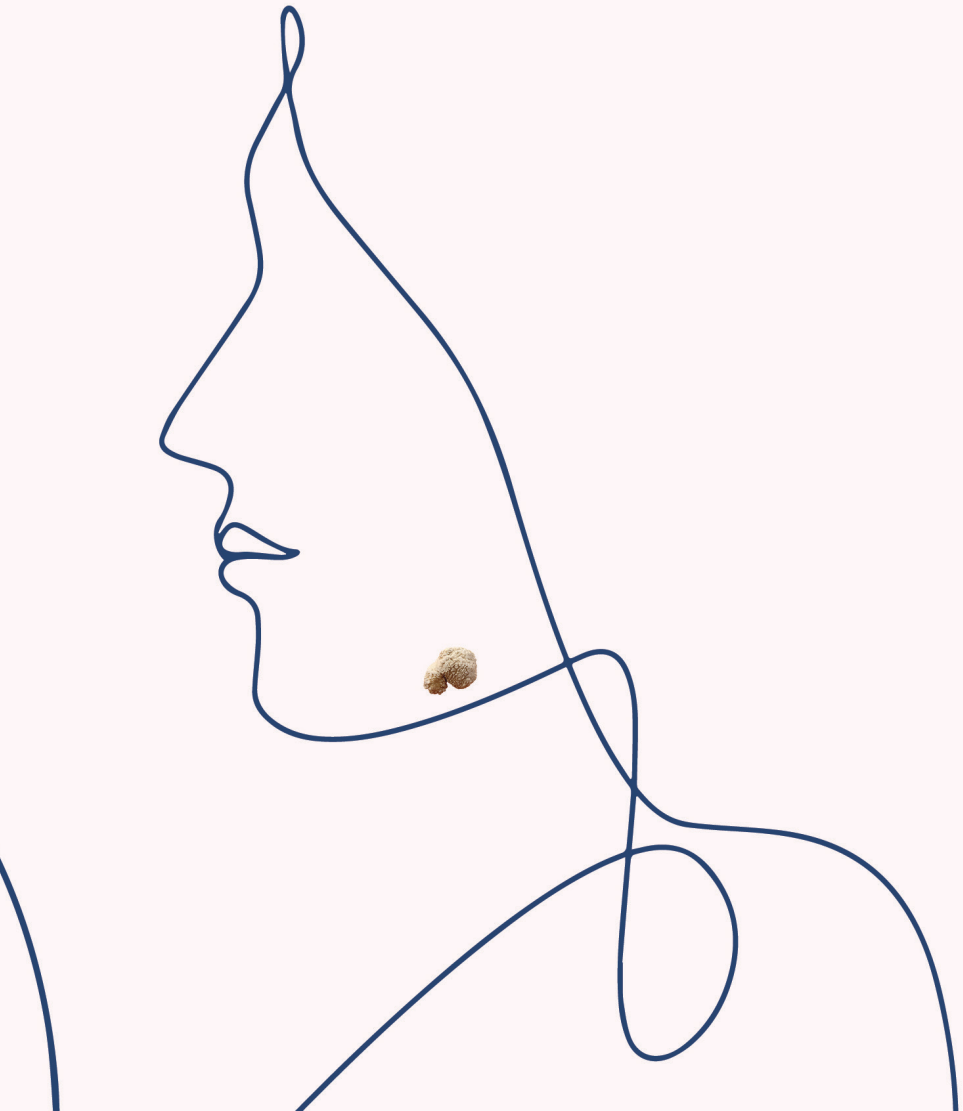

3

CHAPTER

Relationship between volume of submandibular salivary stones in vivo determined with Cone-Beam Computer Tomography and in vitro with micro-Computer Tomography

S Kraaij
HS Brand
EH van der Meij
JGAM de Visscher

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Abstract

Background: Successful removal of salivary stones depends on exact pretreatment information of the location, the size and shape of the stones. This study aimed to compare the volume of submandibular sialoliths determined by preoperative Cone-Beam Computer Tomography (CBCT) scans with the volume of the removed stones on micro-Computer Tomography (micro-CT) scans.

Material and Methods: In this study, using twenty-one submandibular sialoliths, the pretreatment volumes in-vivo measured on CBCT were compared to the volumes of removed stones determined by micro-CT scans. The volume measured om micro-CT scans served as the *gold standard*. Pre-operative CBCT's and in-vitro micro-CT's were converted into standard tessellation language models (STL-models) using an image segmentation software package. The CBCT and micro-CT images of the stones were subsequently metrologically assessed and compared to each other using reverse engineering software.

Results: Volumes of submandibular sialoliths determined by CBCT's correlated significantly with volumes measured on micro-CT's (Spearman's coefficient r = 0.916). The interquartile range (IQR) for the volume measured with micro-CT was 117.23. The median is 26.41. For the volume measured with CBCT the IQR was 141.3 and the median 36.61. The average volume on micro-CT is smaller than on CBCT.

Conclusion: When using CBCT-scans for the detection of submandibular sialoliths one should realize that in-vivo those stones are actually a fraction smaller than assessed on the preoperative scan. This is important when cut-off values of sizes of stones are used in the pretreatment planning of stone removal.

*Keywords***:** *salivary stone; sialolith; CBCT; micro-CT; volume*

Introduction

Salivary stones are mineralized structures most often located in the efferent ducts of the submandibular and parotid glands and less often in the salivary gland itself. This may cause, frequently mealtime related, obstruction resulting in stasis of saliva causing recurrent swelling and pain of the affected salivary gland. In some persistent cases a bacterial sialoadenitis occur (1). Distribution of sialolithiasis in a large series, showed that 80% were located in the submandibular duct system (53% proximal/hilar, 37% distal, 10% intraparenchymal) and 20% in the parotid duct system (83% Stenson's duct, 17% intraparenchymal) (2). For successful treatment of sialolithiasis, exact pretreatment information on the size, volume and location of the salivary stone are important so an informed choice can be made with regard to the most suitable treatment modality. Over 50% of salivary stones cannot clinically be reliable assessed by palpation and/or location (2). Depending on the degree of calcification, some salivary stones can be identified as a radiopaque structure during radiographic examination, despite the relatively high percentage of inorganic material. Various imaging techniques are used to detect the possible presence of salivary stones in patients with recurrent obstructive disease of the submandibular or parotid gland such as occlusal radiograph, panoramic radiograph, sialography, ultrasonography (US), spiral computed tomography (CT) and cone-beam CT (CBCT) and magnetic resonance sialography. CT and CBCT scans are nowadays the preferred radiographic examination techniques for detecting the possible presence of salivary stones with a reported high sensitivity and specificity (3), whereby CBCT is more routinely practice because of the smaller radiation dose (30-80μSv) and lower purchasing costs (4). Micro-CT is basically a miniaturized version of a CT device optimized for the micron imaging but cannot be used for diagnostic examination because of the small scanning range.

The aim of the present study was to compare the volume of salivary stones determined by preoperative CBCT scans with the volume of the removed stones on micro-CT scans in series of submandibular sialolithiasis.

Materials and Methods

In the period from February 2013 to June 2016, in a consecutive series of patients at the department of Oral and Maxillofacial Surgery of the Medical Centre Leeuwarden, the Netherlands, there were twenty-one patients with submandibular salivary stones who had undergone a pretreatment CBCT scan, in an upright sitting position. The CBCT images were performed on a Vatech Panoramic X-Ray System PaX-Zenith 3D radiographic imaging device (*Vatech, Gyeongg-do, Korea)*. The scanning parameters were set at 105kV and 4,5mA. In all cases a large field of view was used. The basic magnification (1,338) of the device when using a large field of view is automatically corrected by the accompanying software making the values of size and shape on the scans correspond to reality.

Before micro-CT imaging, the obtained stones were precisely placed in a medical glove and fixed using polyether impression material Impregum™ Penta™ *(Pentamix 3, 3M ESPE, Seefeld, Germany)*. The fixed salivary stones were scanned using a micro-CT scanner, µCT 40 Scanco Medical *(Wangen-Brüttisellen, Switzerland*. The calculated micro-CT volume served as a 'gold standard' since the accuracy of a micro-CT device is very high (5).

All measurements obtained from the CBCT and micro-CT images were calculated using OsiriX (*Pixmeo SARL, Bernex, Switzerland*) and converted into 3D standard tessellation language (STL) file format surface models. The STL models were subsequently imported into GOM Inspect reverse engineering software (*GOM GmbH, Braunschweig, Germany*) where the distortion was removed and the volume and surface of each stone was measured. In a last step, all CBCT and their corresponding micro-CT STL models were superimposed on each other using GOM software to assess volume differences between the CBCT and micro-CT images.

Statistical analysis was performed with IBM SPSS Statistics for Windows version 26.0 (IBM Inc, Armonk, NY), using Wilcoxon signed rank test and Spearman's rank order coefficient. P-values of 0.05 or less were considered statistically significant.

The current study followed the principles of the Helsinki Declaration and was performed in accordance with the guidelines of the Medical Ethic Committee of the Amsterdam UMC location VUMC (protocol number 2012/127).

Results

Sialoliths were derived from 14 females and 7 males with a mean age of 37 years (range 12-79). Fourteen sialolithiasis were located in the left and 7 in the right submandibular

ductal system. The stones were removed by conventional surgery (7), sialendoscopy (10) and sialendoscopicaly assisted surgical approach ('combined approach') (4). The characteristics of the study population are reported in Table 1. The mean volume of the 21 submandibular salivary stones on CBCT was 141,7 mm^3 (range 8.1 - 840 mm^3) with a median of 36.61 and an interquartile range of 141.3, which was significantly larger than the mean volume on micro-CT of 103,5 mm^3 (range 4.5 - 619.1 mm^3, median 26.41 and IQR 117.23). (Wilcoxon test p = 0.001). On average, submandibular stones measured 19.7% smaller on micro-CT than on the pre-operative CBCT. The volumes determined by CBCT correlated highly significant with the volumes determined with micro-CT (Spearman's coefficient r = 0.916, $p < 0.0005$) (Fig. 1).

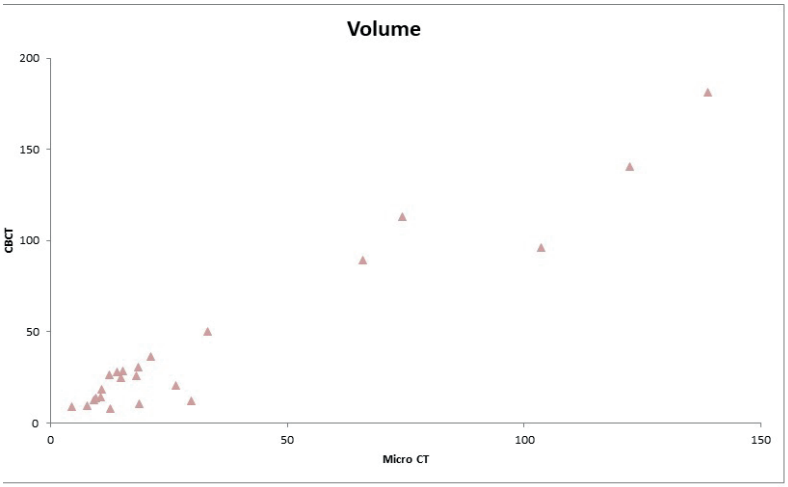

Figure 1 - Relation between the volume determined by both CB-CT and micro-CT of 21 submandibular sialoliths. Data are expressed as mm^3.

Discussion

Exact pretreatment information on the location, size, volume and shape of a salivary stone is essential to guide management and a wide variety of imaging modalities are available for this purpose e.g. MRI, extra oral OPT or conventional X-ray. Each of these imaging modalities has its own advantages and disadvantages with regard to the use of ionizingan imaging modality with high specificity and positive predictive value, and even higher sensitivity and negative predictive value. This high accuracy combined with low costs,

Sample	Gender	Location	Side	Surgical procedure	Volume µCT	Volume CBCT
1	M	D	R	S	103.70	95.96
3	F	D	L	SC	14.13	28.11
5	F	D	R	SC	18.12	26.09
6	F	D	L	SC	330.55	427.88
7	F	D	L	S	10.57	14.36
8	F	D	L	SC	12.31	26.47
10	F	D	R	SC	12.69	8.12
11	M	D	L	SC & S	66.05	89.27
12	F	D	R	SC	10.76	18.52
15	M	D	L	SC	21.08	36.61
16	M	H & D	R	SC & S	122.32	140.49
19	F	D	L	S	138.95	181.25
21	F	H	L	S	619.07	839.95
22	F	D	L	SC	4.50	9.03
24	F	D	L	SC & S	15.18	28.27
27	F	D	L	SC	33.18	50.24
30	M	H	L	S	258.94	504.42
31	M	D	R	SC	18.72	10.54
32	M	D	L	S	26.41	20.63
33	F	H	L	S	262.14	305.79
34	F	D	R	SC & S	74.36	112.99

Table 1 - Characteristics of the study population. Volumes are in mm^3. Abbreviations: D=duct, H=hilus, S=surgical, SC=sialoendoscopy

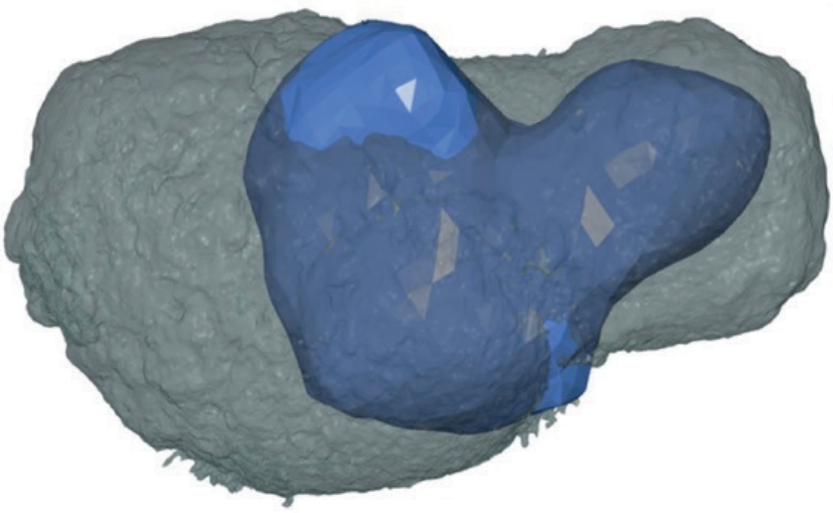

Figure 2 - MUCT (blue, volume 258,94mm^3) and CBCT (gray, volume 504,42mm^3) STL-models projected on top of each other.

high availability and limited radiation exposure makes CBCT an ideal first line imaging modality in patients with signs and symptoms of obstructed major salivary glands (3). US sensitivity for salivary stone detection is assumed to be around 75% (7). Failure has been reported in cases of small and semi-calcified stones. Calculi with a diameter of less than 3 mm are most often missed at US because they do not produce a dorsal acoustic shadow or because they are not hyperechoic with regard to surrounding structures. The lack of a dorsal acoustic shadow may depend not only on the size but also on the chemical composition of calculi. Besides, calculi within the distal duct are not shown accurately with US. Recently it was reported that ultrasound measurements of salivary stones in millimeters correlated highly with ex vivo measurements after removal (7,8). Conventional 2D radiography is still routinely used in daily practice nowadays. However, on panoramic radiographs, salivary stones can be missed because they may be projected superimposed on bony structures or teeth. In addition, occlusal and panoramic radiographs are two-dimensional imaging modalities, with concomitant limited possibilities to determine the volume and shape of the sialoliths (1,9).

Previous studies suggest that submandibular stones with a diameter of less than 4 mm may be manageable to sialendoscopical removal (10,11,12). Unfortunately, the practical value of the current used cut-off value is limited, due to the use of various imaging techniques and the fact that none of the studies indicated whether the cut-off diameter concerned the widest cross section or the longitudinal section.

The results of the present study suggest that when CBCT-scans are used for the detection of submandibular salivary stones one should realize that in vivo those stones are actually a fraction smaller than assessed on the preoperative CBCT-scan. This finding is particularly important when cut-off values of sizes of stones are used in the pretreatment planning of stone removal. A possible limitation of this study is the setting of the voxel size on the CBCT device. Volume measurements up to a voxel size of 200mm (100mm, 150mm and 200m) show no differences in measurements, despite a slight tendency towards underestimation, which increases with voxel size. At 300mm and above, the underestimation of measurements becomes statistically significant (13,14). To overcome this limitation and to ensure that one measures the actual volume of the stone, it is recommended to use the smallest voxel size possible.

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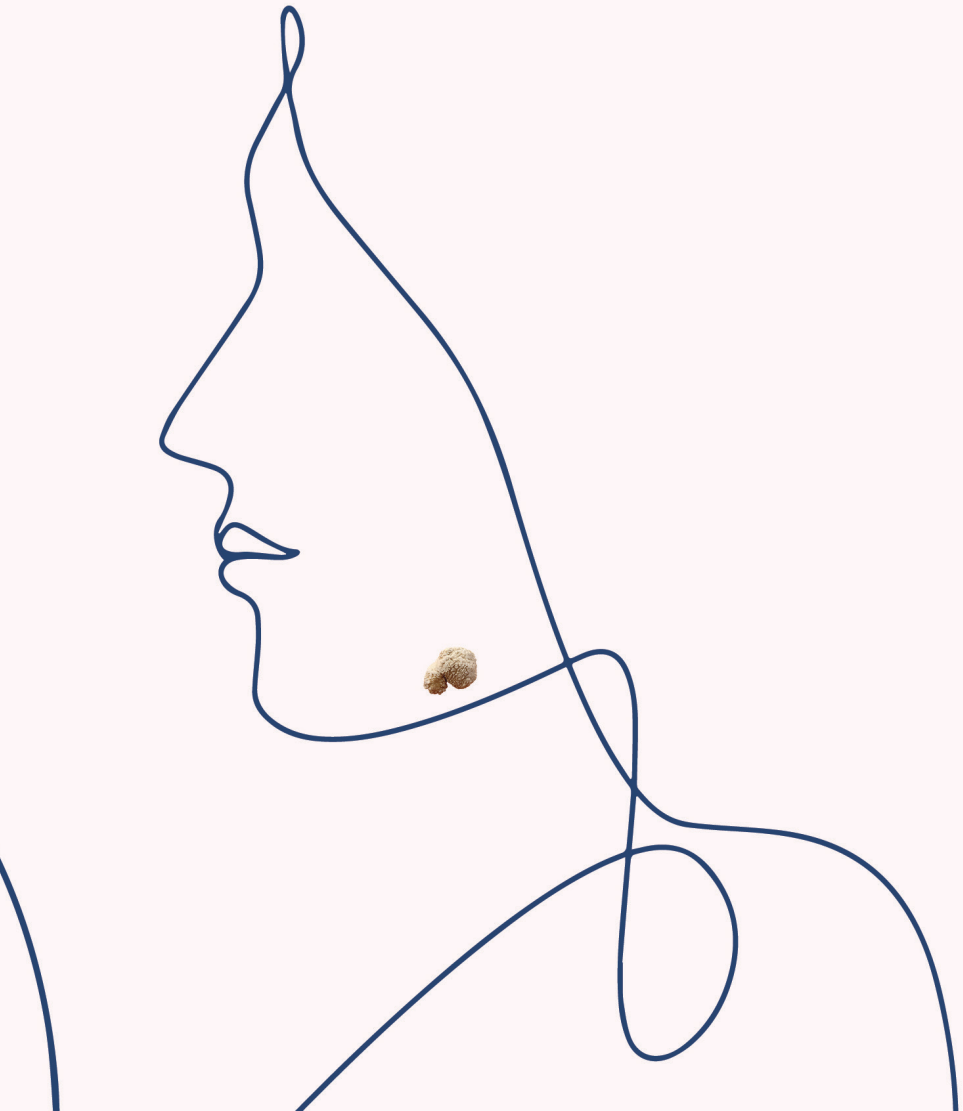

4

CHAPTER

Systemic diseases and the risk of developing salivary stones: a case control study

S Kraaij
KH Karagozoglu
YA Kenter
J Pijpe
M Gilijamse
HS Brand

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ABSTRACT

Objective: To investigate the possible relationship between the presence of salivary stones and systemic diseases, medication, smoking and alcohol consumption.
Study design: A retrospective, case-control study.
Materials and Methods: Medical records of patients with salivary stones and control patients without salivary stones were retrospectively reviewed. Data regarding the affected salivary gland, the presence of systemic disease, the use of medication, tobacco and alcohol were recorded. Statistical analysis was performed using Fisher's exact tests.
Results: Medical records of 208 patients with salivary stones and 208 control patients were reviewed. Of the patients diagnosed with salivary stones, the submandibular gland was affected in 85.6% of the patients, the parotid gland in 9.6% and the sublingual gland in 2.4% of the patients. None of the recorded systemic diseases were more prevalent in patients with salivary stones. Patients with salivary stones used significantly more antibiotics than the control group ($p = 0.037$). No significant differences were observed for other types of medication. There was no correlation between salivary stone formation, smoking and alcohol consumption.
Conclusion: The present study suggests that systemic diseases, medication, smoking and alcohol consumption play no or only a limited role in the onset of salivary stones.

INTRODUCTION

Salivary stones are present as one or more calcified structures within the salivary ductal system. The formation of salivary stones, also known as sialoliths, can result in partial or total obstruction of the ductal system leading to recurrent swelling of the salivary gland, pain and acute or chronic infection. The annual incidence of salivary stones is estimated between 1 per 15.000 and 1 per 30.000 individuals.[1] Salivary stones occur most often in patients over 40 years of age and rarely in children.[2] In studies published before 1990 a male predominance is mainly found. However, more recent studies show an almost equal distribution between males and females.[3,4,5,6,7,8,9,10] More than half of the salivary stones have a diameter between 2.1 and 10 mm, and only 7.6% are larger than 15 mm in section.[11] Salivary stones are more often located within the ductal system of the submandibular gland (72-95%) than in the ductal system of the parotid gland (4-28%). Salivary stones are seldom found in the sublingual glands or accessory salivary glands.[5,6,9,10,11] The higher incidence of salivary stones in the ductal system of the submandibular gland is probably caused by its longer duct, salivary flow against gravity and a more alkaline saliva with a higher calcium and mucin content.[9]

The cause of salivary stones formation remains unclear. However, there are two main theories that try to explain the formation of salivary stones. The first theory postulates that a local inflammatory process leads to calcification of a mucus plug. The second theory assumes that micro-sialoliths, produced by autophagosomes in the salivary gland, form a nidus for calcium precipitation.[2] Salivary stones are mainly composed of inorganic material like hydroxyapatite, carbonate apatite, whitlockite and brushite, with smaller amounts of organic material such as collagen, glycoproteins, lipids and carbohydrates.[11] Kidney stones mainly contain calcium oxalate, uric acid, struvite, cystine and small amounts of phosphate and ammonium.[12] The main components of gallstones are cholesterol, bilirubin, bile acids, calcium apatite and small amounts of magnesium and struvite.[13]

Some cases have been described in which individuals developed a salivary stone concomitant with gallstones[14] or kidney stones[15]. Conflicting data were obtained when this possible relation was investigated in groups of patients with salivary stones. Lustmann and co-workers[6] found an incidence of kidney stones of 10.7% in a group of 56 patients with salivary stone formation. This is higher than the incidence in the general population and suggests a relation between salivary stone formation and nephrolithiasis. However, Zenk et al.[10] found that the prevalence of nephrolithiasis (2%) and cholelithiasis (1.6%) in a group of 635 patients with salivary stone formation was not increased compared

to the general population. Huoh et al.[16] retrieved data from medical records of patients diagnosed with salivary stones at the University of California. They also found that the prevalence of cholelithiasis in patients with salivary stone formation was not higher than the prevalence in the general population.

In the studies cited above[6,10,16] the incidence of systemic disorders in patients with salivary stone formation was compared with data on the prevalence of diseases in the general population obtained from other studies. To our knowledge, no case-control studies have investigated the potential association between systemic disorders and the development of salivary stones. Therefore we performed a case-control study to explore the relationship between the onset of salivary stones and the presence of systemic diseases, use of medication and lifestyle factors.

Materials and Methods

A retrospective case-control study was conducted. The case group consisted of patients with salivary stones who had undergone surgical removal of sialoliths in the VU University Medical Center Amsterdam, Catharina Hospital in Eindhoven and Onze Lieve Vrouwe Gasthuis in Amsterdam in The Netherlands from November 1, 2001 until December 31, 2013. All patients had been referred to the hospitals because of symptomatic salivary stones. A total of 208 medical reports of patients with salivary stones were available for analysis. Each patient with a salivary stone was matched with an age- and control matched individual presenting at the same departments of Oral and Maxillofacial Surgery with medical problems other than a salivary stone.

The medical records of both the case patients and the matched control patients were systematically reviewed. The following clinical data were retrieved and processed anonymously into an Excel spreadsheet: the affected salivary gland and the presence of hyperthyroidism, diabetes, cardiovascular disease, hypertension, Human Immunodeficiency Virus (HIV), tuberculosis, cholelithiasis, nephrolithiasis, cirrhosis, hepatitis, Parkinson's disease, epilepsy, rheumatoid arthritis, Sjögren's syndrome, gout and malignancies. Data about medication use were obtained and categorized according to the Dutch national formulary (Pharmacotherapeutisch Kompas)[17]. The records were also analyzed for information about currently smoking tobacco and/or use of alcohol. When data on smoking or use of alcohol were available in the patients' records, these data were processed as yes/no. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Inc, Armonk, NY), using Fisher's Exact 2-sided tests. P values < 0.05 were considered significant.

Results

A total of 208 patients with salivary stones, 112 males (54%) and 96 females (46%) (male:female ratio, 1.17:1), mean age of 46.8 years (range 8-87 years) were identified. A case-control group consisting of 208 patients, 112 males and 96 females, mean age 46.4 years (range 8-88 years) was created. The submandibular gland was affected in 85.6% of the patients, the parotid gland in 9.6% and the sublingual gland in 2.4%. The prevalence of systemic diseases in both patient and control group is presented in Table 1.

	Sialolithiasis	Control	p-value
Endocrine diseases			
Hyperthyroidism	0 (0%)	1 (0.5%)	1.000
Diabetes	9 (4.3%)	6 (2.9%)	0.600
Cardiovascular diseases			
Hypertension	21 (10.1%)	23 (11.1%)	0.874
Cardiovascular disease (other than hypertension)	19 (9.1%)	19 (9.1%)	1.000
Infectious diseases			
Human Immunodeficiency Virus (HIV)	0 (0%)	0 (0%)	---
Tuberculosis	1 (0.5%)	0 (0%)	1.000
Hepatitis	2 (1%)	2 (1%)	1.000
Internal diseases			
Cholelithiasis	0 (0%)	5 (2.4%)	0.061
Nephrolithiasis	3 (1.4%)	3 (1.4%)	1.000
Cirrhosis	0 (0%)	1 (0.5%)	1.000
Neurologic diseases			
Parkinson's disease	0 (0%)	0 (0%)	---
Epilepsy	2 (1%)	1 (0.5%)	1.000
Rheumatologic diseases			
Rheumatoid arthritis	1 (0.5%)	4 (1.9%)	0.372
Sjögren's syndrome	0 (0%)	5 (2.4%)	0.061
Gout	0 (0%)	0 (0%)	---
Malignant diseases	8 (3.8%)	12 (5.8%)	0.493

Table 1 - Prevalence of systemic diseases in patients with salivary stone formation and control subjects (both n= 208)

No relation was found between salivary stone formation and most systemic diseases. However, the prevalence of cholelithiasis and Sjögren's syndrome was almost significantly higher in the control group than in patients with salivary stones (p=0.061).

There was a lack of data regarding smoking in 72% of the patients with salivary stone formation and in 51% of the control subjects. In the available documents, however, there was a trend toward statistical significance (p = 0.097) with 49.2% smokers amongst 59

patients with salivary stone formation and 34.9% smokers amongst 106 control subjects. Data regarding alcohol use were lacking in the records of 76% of the patients with salivary stone formation and 57% of the control subjects. The available data did not show a significant difference in alcohol consumption between the 50 individuals with salivary stone formation (50%) and the 90 individuals from the control group (55.6%) ($p = 0.597$).

Discussion

Several studies have investigated the possible relation between salivary stone formation and systemic diseases or use of medication. Lustmann and co-workers[6] found an incidence of kidney stones of 10.7% in patients with salivary stone formation, which was considerably higher than the incidence in the general population. In our case-control study the prevalence of nephrolithiasis in the patient group did not differ from the prevalence in the control group. These results are similar to those in the studies of Zenk et al.[10] and Huoh et al.[16] who reported prevalence rates of kidney stones comparable to the general population. This suggests that the risk factors for developing a salivary stone and a kidney stone are not related.

An Italian study reported high prevalence rates of diabetes mellitus (25%) and hypertension (20%) in patients with salivary stone formation.[4] However, in a subsequent study the prevalence of hypertension and diabetes mellitus was comparable to the prevalence in the general population.[10] In our case-control study, we also observed no differences in the prevalence rates of hypertension and diabetes mellitus (Table 1).

Leung et al[14] described a 49 year old man with multiple salivary stones and a medical history of multiple gallstones. We observed a non-significant higher prevalence of cholelithiasis in the control group than in the patients with salivary stone formation (Table 1). In a previous study, the prevalence of cholelithiasis in a group of 635 patients with salivary stone formation was not higher than in the general population[10], which was confirmed by Huoh et al[16].

It is hypothesized that a decreased salivary flow rate may facilitate the formation of salivary stones.[2] However, we did not find an increased prevalence of salivary stones in patients with Sjögren's syndrome. The relatively high prevalence of Sjögren's syndrome in control subjects in the present study (Table 1), is probably explained by the fact that patients with a suspicion of Sjögren's syndrome are frequently referred to an Oral and Maxillofacial surgeon for further investigation and subsequently are overrepresented in the control group.

Two case reports suggested a possible relation between salivary stone formation and medication. Perrotta et al.[18] described a 57-year old woman with Parkinson's disease and salivary stones in both submandibular and parotid gland, who was treated with levodopa and amantadine. The authors suggested that medication of this patient may have contributed to sialolith formation. Another case report[19] described a 76-year old woman with salivary stones in both submandibular glands with a medical history of hypertension and a myocardial infarction two years earlier and use of methyldopa.

Many types of medications such as diuretics, antihistamines, antihypertensive drugs, antipsychotic medications and antidepressants decrease the salivary flow rate. Subsequently use of these medications could facilitate formation of salivary stones.[9] Diuretic use in the cohort group of Huoh and co-workers[16] was higher than the use of diuretics in the general population. In the present case-control study the use of diuretics in patients with salivary stone formation was comparable with the control group (Table 2). This in agreement with the study of Zenk et al.[10], where the use of diuretics by patients with salivary stones formation was comparable to the general population. Our case-control study also confirms the study of Zenk et al.[10] that use of thyroid medication and anti-diabetic medication by patients with salivary stone formation is comparable to individuals without a salivary stone.

The use of antibiotics was higher in the group of patients with salivary stone formation compared with the control group (Table 2). This may be related to the fact that salivary stone formation is frequently associated with sialadenitis[20]. In many cases, the referring dentist or general practitioner may have been prescribed antibiotics as initial treatment.

Huoh et al[16] found a higher rate of smoking or history of smoking in patients with salivary stones than in the general population, although the difference did not reach statistical significance. In our case-control study we observed a similar trend with more smokers amongst patients with salivary stone formation, suggesting that smoking increases the risk of developing a salivary stone. Tobacco smoking can cause inflammation, resulting in subsequent formation of a mucus plug and intraglandular concretion, ultimately resulting in the formation of a salivary stone.[9,20]

The mean age of the patients with salivary stone formation in the present study is comparable to that of previous studies.[5,6,10,16] The distribution of salivary stones over the different salivary glands is also in accordance with previous reports.[5,9,18,20,21] In the present study, a small male preponderance was observed, which is in agreement with the study of Zenk et al.[10] Older studies found a more extensive male preponderance.[4,5]

	Sialolithiasis	Control	p-value
Cardiovascular medication	44 (21.5%)	40 (19.2%)	0.714
Diuretics	16 (7.7%)	14 (6.7%)	0.850
Anti-diabetic agents	8 (3.8%)	9 (4.3%)	1.000
Stomach medication	21 (10.1%)	23 (11.1%)	0.874
Rheumatism medication	0 (0%)	2 (1%)	0.499
Analgesics	7 (3.4%)	13 (6.3%)	0.251
Anti-inflammatory drugs	18 (8.7%)	21 (10.1%)	0.737
Anti-epileptics	3 (1.4%)	2 (1%)	1.000
Migraine medication	1 (0.5%)	3 (1.4%)	0.623
Respiratory medication	19 (9.1%)	16 (7.7%)	0.724
Antidepressants	6 (2.9%)	11 (5.3%)	0.322
Anxiolytics	4 (1.9%)	2 (1%)	0.685
Oral contraceptives	5 (2.4%)	5 (2.4%)	1.000
Thyroid medication	2 (1%)	4 (1.9%)	0.685
Bisphosphonates	2 (1%)	7 (3.4%)	0.175
Antihistamines	9 (4.3%)	9 (4.3%)	1.000
Antipsychotics	1 (0.5%)	3 (1.4%)	0.623
Benzodiazepines / Hypnotics	2 (1%)	3 (1.4%)	1.000
Antibiotics	8 (3.8%)	1 (0.5%)	0.037
Dietary supplements	10 (4.8%)	14 (6.7%)	0.529

Table 2 - Medication used by patients with salivary stone formation and control subjects (both n=208)

A potential limitation of the present case-control study is that data of the medical records were used, which were not specifically registered for scientific purposes. Incompleteness of information in medical history records is rather common.[22] Another potential limitation is the hospital based character of the study. The control subjects also have been referred to the departments of Oral Maxillofacial Surgery for specific diseases, such as oral malignancies and Sjögren's disease. This means that systemic factors that are associated with these oral diseases may have been overrepresented in the control group, thereby obscuring the potential contribution of certain systemic factors in developing salivary stones.

Despite these limitations, the data of the present case-control study indicate that systemic diseases and use of medication do not play a prominent role in the development of salivary stones. This suggests that local factors like anatomical variations of the salivary ducts and/or an altered biochemical composition of saliva are probably more important factors in the development of salivary stones.[23]

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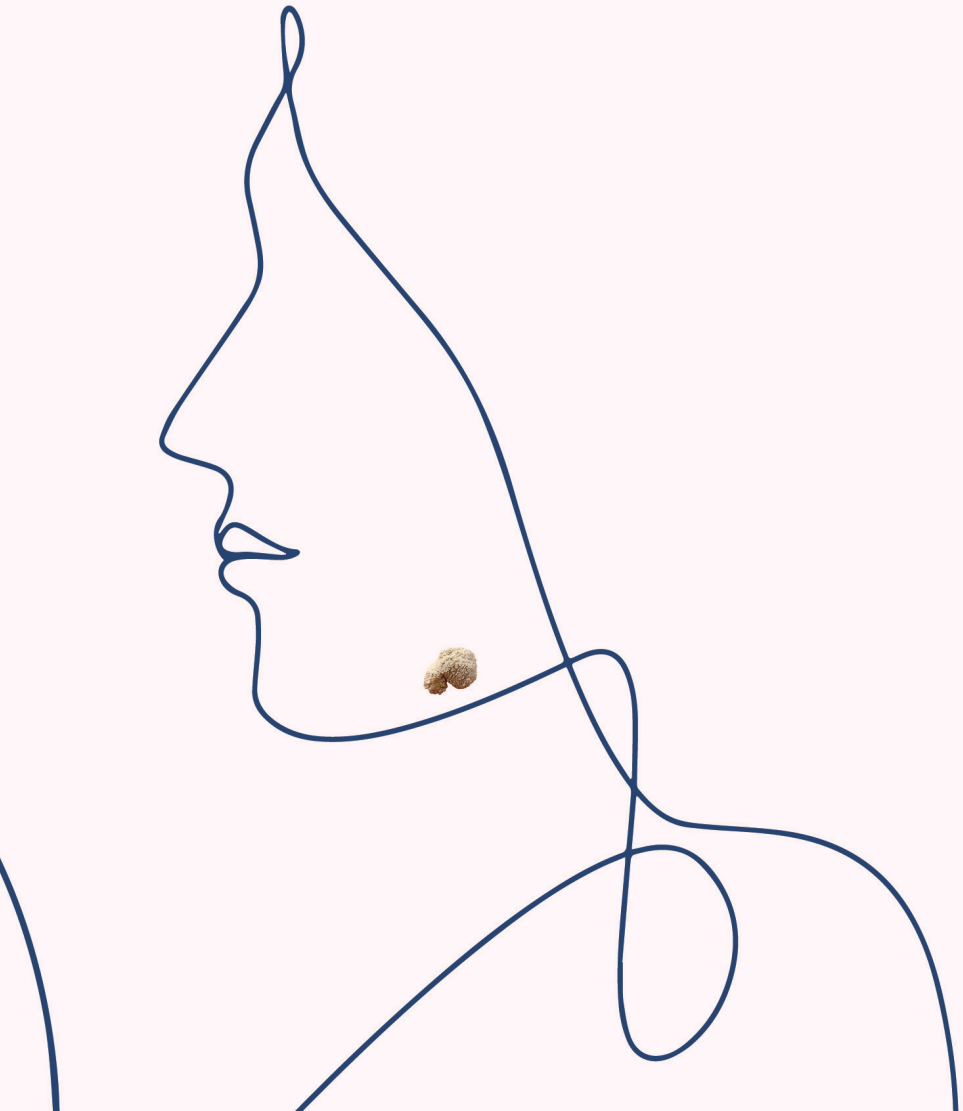

5

CHAPTER

Biochemical composition of salivary stones in relation to stone- and patient-related factors

S Kraaij
HS Brand
EH van der Meij
JGAM de Visscher

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ABSTRACT

Background: Salivary stones are calcified structures most often found in the main duct of the submandibular or parotid salivary gland. They contain of a core surrounded by laminated layers of organic and inorganic material.

***Materials and Methods*:** Submandibular and parotid sialoliths (n=155) were collected at the department of Oral and Maxillofacial surgery of a general hospital between February 1982 and September 2012. The weight of the sialoliths was determined and the consistency was subjectively classified. Subsequently, the biochemical composition of the stones was determined by wet chemical methods or FT-IR spectrometry. Age and gender of the patients were retrieved from their medical records. Data were statistically analyzed using Fisher's exact tests.

***Results*:** Sialoliths are mainly composed of inorganic material. Carbonate apatite was identified in 99% of the stones, phosphate in 88%, calcium in 87%, magnesium in 68%, struvite in 44%, oxalate in 38% and carbonate in 35%. Solid salivary stones contain more frequently struvite than stones with a soft consistency (p=0.05). Larger stones (>100mg) contain more frequently carbonate (p=0.05). Stones from older patients (≥38years) showed an almost significant trend towards more frequent presence of phosphate (p=0.083).

***Conclusion*:** The biochemical composition of submandibular and parotid sialoliths is related to stone-related factors, probably to age but not to the gender of the patient.

INTRODUCTION

Salivary stones are calcified structures most often found in the main duct of the submandibular or parotid salivary gland, which may cause mechanical obstruction associated with stasis of the saliva in the duct and gland. Associated symptoms are (mealtime related) recurrent swelling, pain and sometimes as a result, inflammation of the gland. Salivary stones, also called sialoliths, are most frequently located in the submandibular duct and salivary gland (72-95%) and less frequently in the parotid duct and gland (4-28%).[1] The sublingual and minor salivary glands are rarely affected. The mean annual incidence of hospital admission for patients with symptomatic sialolithiasis in the United Kingdom varies between 27.5 and 59 per million population per annum.[2] Sialolithiasis is most common in patients in the fourth and fifth decade of life and is equally distributed between men and women.

The etiopathogenesis of salivary stones is not completely understood. There are three main theories: agglomeration of sialomicroliths, calcification of a mucus plug and an altered biochemical composition of saliva.[1] Su and co-workers[3] found that the saliva of patients with salivary stones is supersaturated with calcium and unsatiated with citrate, phytate and magnesium. It is assumed that salivary stasis or a decreased salivary flow contributes to the precipitation of calcium.

Submandibular and parotid salivary stones have similar structures. They consist of an amorphous, mineralized core surrounded by concentric laminated layers of organic and inorganic material. A very small percentage of sialoliths, submandibular as well as parotid, only consist of a core. The diameter of the nucleus varies between 0.5 and 1.5mm and is usually homogeneous but may contain substructures. These substructures refer to the proposed pathogenesis of sialoliths by agglomeration of sialomicroliths. These differences in structure and build-up may cause differences in colour and hardness of salivary stones.[4,5]

The composition of salivary stones can be analyzed with different techniques: wet chemical techniques, X-ray powder diffraction and/or infrared spectroscopy (FT-IR).[4,6] X-ray diffraction and infrared spectroscopy offer the best identification of components, are fast and reproducible whereby infrared spectroscopy is becoming the gold standard.[7,8] The infrared spectrum originates from the vibrational motion of the molecules. The vibrational frequencies are a kind of fingerprint of the compounds. This property is used for the characterization of organic and inorganic compounds present in calculi.

Knowledge of the biochemical composition of salivary stones is essential for understanding their etiology. Therefore, the aim of the present study was to investigate whether the inorganic biochemical composition of salivary stones is related to stone-related factors (size, consistency) and / or patient-related factors (age and gender of patient).

Materials and methods

Patients and stones

In the period from February 1982 to June 1996 the department of Oral and Maxillofacial Surgery of the Medical Centre Leeuwarden obtained 67 salivary stones from 67 patients (36 men and 28 women, 3 gender unknown) (group 1). The mean age of these patients was 37 years (age range 4-79). All these stones were from ducts of submandibular salivary glands. Between March 1997 and September 2012 another series of 88 salivary stones from 87 patients (45 men and 41 women, 1 gender unknown) was collected (group 2). The mean age of this patient group was 47 years (age range 8-87). Nine of the salivary stones from this series were from the parotid gland (10%) and 69 from the submandibular salivary gland (78%), the origin of ten stones was not registered. 36 percent of the salivary stones in group 2 were from the left and 52 percent from the right side.

All stones were removed through sialendoscopy, a transoral approach or by surgical removal of the affected gland. After removal, the stones were washed with distilled water and stored in plastic jars.

During the course of this study, all guidelines and protocols of the Declaration of Helsinki were followed.[9]

Stone analysis

Salivary stones collected in the first period (group 1) were quantitative analyzed using wet chemistry methods as described by Larsson et al. (1984).[10] Wet chemical analysis is based on the quantification of ions and organic components, from which the quantitative composition of the components can be calculated. Ions and organic material such as calcium, magnesium, ammonium, oxalate, phosphate, carbonate, urate and cystine can be detected.[11] Stones obtained in the second period (group 2) were subjectively classified by an Oral and Maxillofacial surgeon as solid or soft. Subsequently, they were analyzed by Fourier Transform Infrared spectrometry (FT-IR) (mid infrared region 4000-400 cm^{-1}) using the KBr disk technique.[12,18] The size of a peak in the spectrum corresponds exactly with the quantity of a specific compound. Qualitative estimations of the presence of whewelliet,

wheddeliet, carbonate apatite, struvite, brushite, cystine, ammonium urate and proteins were obtained.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 24.0 (IBM Inc, Armonk, NY), using Fisher's Exact 2-sided tests and Spearman's rank order coefficient. P values < 0.05 were considered statistically significant.

Results

The salivary stones collected during the first period had an average weight of 418mg (s.d. 1278). The weight of the salivary stone correlated significantly with the age of the patient ($r = 0.382$, $p = 0.002$) (Figure 1). The components in these salivary stones, identified by wet chemical analysis, are presented in Table 1. Most of the stones contained phosphate (88.4%), calcium (87.0%) and magnesium (68.1%). Carbonate and oxalate were present in approximately one third of the stones. Ammonium, cystine and urate were rarely detected (<3%). The biochemical composition of the salivary stones collected during the second period was determined using FT-IR. Nearly all these salivary stones contained carbonate apatite (98.9%) and in approximately a half of the stones struvite was present (43.7%). Wheddeliet (9.2%), whewelliet (3.5%), brushite (5.6%), ammonium urate (1.2%) and proteins (1.2%) were rarely identified. Cystine was not detected in any of the salivary stones.

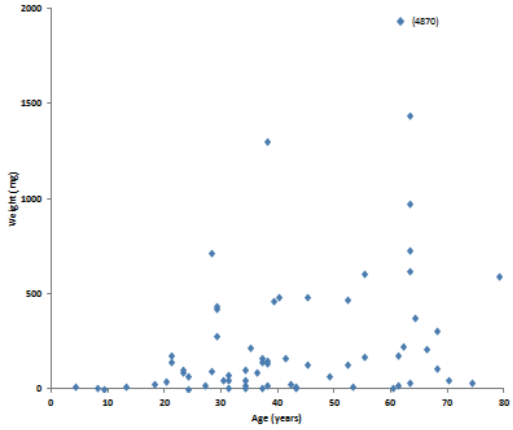

Figure 1 - Relation between weight of the salivary stone and the age of the patient Spearman rank order coefficient, (r = 0.382, p = 0.002) (n=66)

The salivary stones from group 1 were dichotomized, based on the median weight of 100 mg, into two groups: 'low weight' (≤100mg, n=32) and 'high weight' (≥ 101mg, n=35). Stones from the 'high weight' group contained more frequently magnesium and carbonate than stones from the 'low weight' group. Percentages oxalate and phosphate were almost equal for both groups. (Table 1)

Based on the median age, the patients from the first period were also stratified in two groups: 'young' (≤ 37 years, n=29) and 'old' (≥ 38 years, n=35). Stones from 'old' patients contained more phosphate than stones from the 'young' group. This difference almost reached statistical significance (p=0.083) (Table 1). The stones collected during the second period were also stratified according to the mean age (47 years) into 'young' (≤ 47 years, n=44) and 'old' (≥ 48 years, n=43) No age-related differences were observed in the biochemical parameters determined by FT-IR. (Table 2)

Gender of the patient had no significant effects on the biochemical composition of salivary stones. (Table 1 and 2)

Stones collected during the second period were subjective classified as 'hard' or 'soft'. Salivary stones classified as hard contained more frequently struvite than stones from the 'soft' group (p=0.005). (Table 2)

Discussion

Insight in the biochemical composition of salivary stones might provide information to clarify the etiopathogenesis of salivary stones, to facilitate diagnosis, to prevent formation and to improve treatment. The present study has shown that the biochemical composition of salivary stones is related to stone-related factors as size and consistency.

Larger stones contain more frequently carbonate (Table 1). This might be related to the growth of sialoliths, where an initial amorphous core becomes gradually surrounded by concentric laminated layers. These surrounding layers contain carbonate apatite, and differ in degree of mineralization.[1] During the growth of the sialolith the number of laminated layers will increase, which might explain the increased contribution of carbonate in larger sialoliths. The weight of the sialolith is significantly related to the age of the patient (Figure 1). This might relate to age-related changes in circulating serum levels of phosphate. Several studies have reported that the serum phosphate levels are significantly lower in adults above the age of 50 years.[13,14] Phosphate acts as crystallization inhibitor.[15]

	All (n=69)	≤100mg (n=32)	≥101mg (n=35)	p-value[1]	Male (n=36)	Female (n=28)	p-value[1]	≤ 37 yrs (n=29)	≥ 38 yrs (n=35)	p-value[1]
Ca^{2+}	87.0	90.6	88.6	1.000	91.7	85.7	0.689	89.7	88.6	1.000
Mg^{2+}	68.1	56.2	82.9	0.031	75.0	67.7	0.583	72.4	68.6	0.789
NH_4^+	11.6	10.0	14.3	0.716	11.4	14.8	0.719	13.8	9.1	0.696
Oxalate	37.7	40.0	40.0	1.000	45.7	33.3	0.435	29.6	48.6	0.192
Phosphate	88.4	90.6	91.4	1.000	91.7	89.3	1.000	82.8	97.1	0.083
Urate	1.4	3.8	0.0	0.433	3.1	0.0	1.000	3.8	0.0	0.448
Carbonate	34.8	15.6	54.3	0.002	36.1	39.3	0.801	37.9	34.3	0.798
Cystine	2.9	0.0	5.9	0.503	6.3	0.0	0.499	0.0	6.5	0.497

Table 1 - Biochemical analysis of salivary stones, stratified according to size of the stone and gender and age of the patient. The numbers in the table indicate the percentage of the salivary stones that contain the specific component. [1] Fisher's exact test 2-sided

	All (n=87)	Hard (n=34)	Soft (n=40)	p-value[1]	Male (n=46)	Female (n=41)	p-value[1]	≤ 47 yrs (n=44)	≥ 50 yrs (n=43)	p-value[1]
Whewelliet	3.5	5.9	2.5	0.591	4.3	2.4	1.000	2.3	4.7	0.616
Wheddeliet	9.2	11.8	7.5	0.696	8.7	9.8	1.000	9.1	9.3	1.000
Carbonate apatite	98.9	97.1	100	0.459	95.7	100	0.496	100	95.3	0.241
Struvite	43.7	67.6	32.5	0.005	41.3	46.3	0.670	52.3	34.9	0.131
Brushite	4.6	0.0	7.5	0.245	6.5	2.4	0.619	4.5	2.3	1.000

Table 2 – Biochemical analysis of salivary stones, stratified according to hardness of the stone and gender and age of the patient. The numbers in the table indicate the percentage of the salivary stones that contain the specific component. [1] Fisher's exact test 2-sided

Therefore, reduced circulating levels of phosphate could result in less inhibition of crystallization, resulting in larger sialoliths in older individuals. However, this suggestion seems to be contradicted by the biochemical analysis of sialoliths from older individuals. Older individuals showed a trend towards more frequent presence of phosphate in comparison with stones from the 'young' group instead of a reduced presence of phosphate (Table 1). An alternative explanation for the observed association between age and weight of the salivary stone is that in older individuals sialoliths had a longer time to develop.

Brushite was only detected in a relatively small number of salivary stones (Table 2), much lower than the percentage stones containing carbonate and oxalate. This might be related to the fact that brushite dissolves more rapidly than other calcium minerals like calcium carbonate and calcium oxalate.[16]

In the present study, 38% of the salivary stones contained oxalate. Kidney stones are mainly consisting of calcium oxalate, and they occur two to three times more often in men than in women. Watson and co-workers (2010)[17] showed that men with kidney stones have an increased serum total testosterone level, suggesting that this hormone might be related to the deposition of oxalate. To our knowledge, no data are available on the possible relationship between serum total testosterone levels and salivary stones. However, in the present study, salivary stones containing oxalate were more common in men (45.7%) than in women (33.3%), although this difference did not reach statistical significance.

In the present study, protein was detected only in 1% of the salivary stones. This percentage is much lower than previously reported by Sabot and co-workers[6], who identified proteins in more than three quarters of salivary stones. This difference in result could be explained by the fact that the value of infra-red spectrometry in the detection of proteins is rather limited.

The most important limitation of the present study is its retrospective nature, making it dependent on historically collected data. In the eighties of the last century, it was common to use wet chemical techniques for salivary stone analysis because of its low costs. The last 25 years, FT-IR spectrometry for analysis of stones became the analysis of choice. FT-IR spectrometry is a fast and precise technique. The 2013 guidelines on urolithiasis of the European Association of Urology underline the obsolescence of chemical wet analysis and recommend the use of FT-IR for stone analysis.[18]

Despite these limitations, the presented historical data suggest that the biochemical composition of salivary stones is related to stone-related factors as size and consistency.

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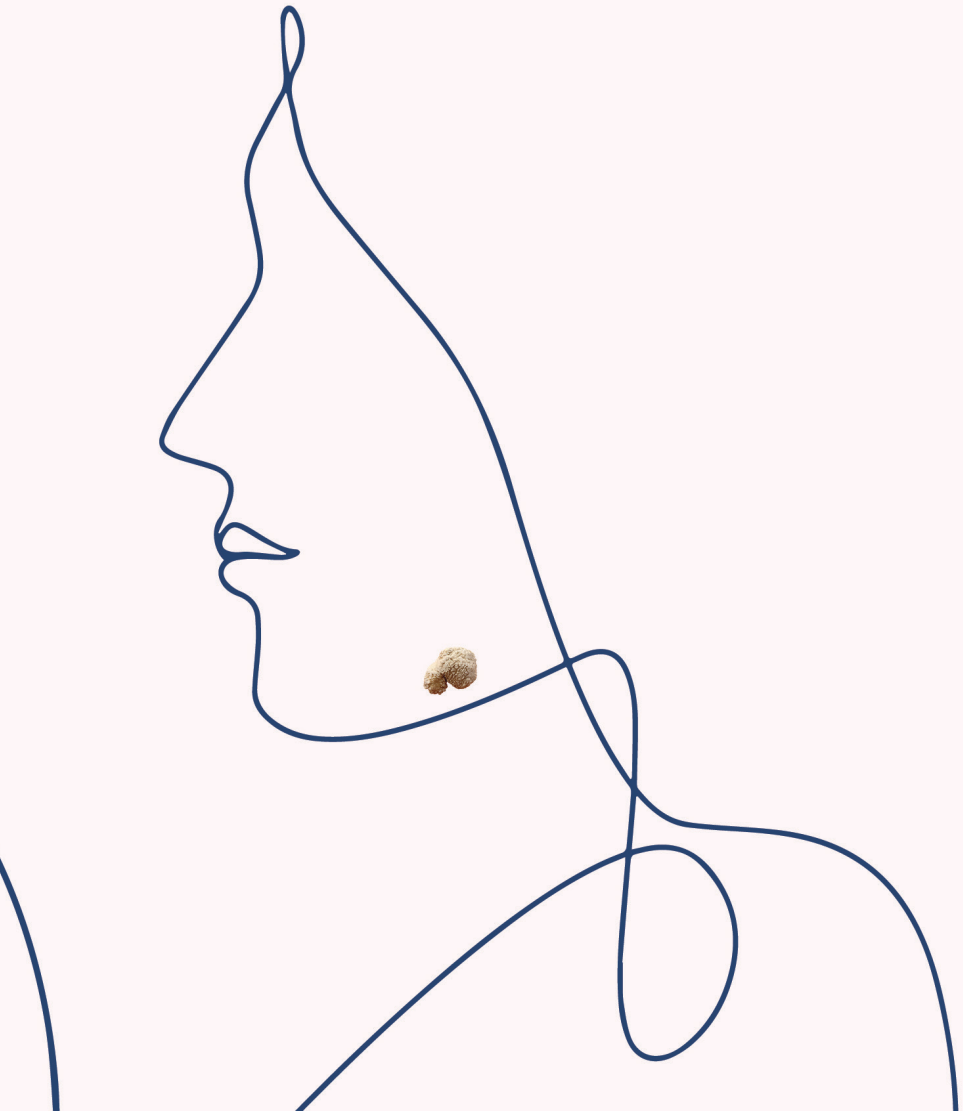

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CHAPTER

Lactoferrin and the development of salivary stones - a pilot study

S Kraaij
JGAM de Visscher
RC Apperloo
K Nazmi
FJ Bikker
HS Brand

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ABSTRACT

Salivary stones (sialoliths) are calcified structures located in the ductal system of the major salivary glands. Their exact cause is not clear but in general they are characterized by concentric inorganic (hydroxyapatite) layers. The formation is a slow intermittent process which may result in enlargement of the sialolith causing obstruction of saliva secretion resulting in mealtime related pain and swelling of the affected salivary gland. Various studies reported the presence of organic material such as proteins and lipids in the core of sialoliths.

In the present study the protein composition of twenty submandibular sialoliths was analyzed. It was found that proteins contributed on average 5% to the dry weight of submandibular stones whereby small salivary stones contained more extractable proteins than large salivary stones. Using a combination of SDS-PAGE gel electrophoresis and Western blotting, we identified α-amylase (in all stones;100%), lysozyme (95%), lactoferrin (85%), secretory-IgA (75%), MUC7 (60%), complement C4 (60%) and C-reactive protein (35%). The presence, and the combinations, of lactoferrin, lysozyme, s-IgA and α-amylase in sialoliths was confirmed by ELISA. The gradually increasing size of a sialolith might provoke a local inflammatory response in the duct of the submandibular gland whereby the relatively low concentrations of lactoferrin and lysozyme may originate from neutrophils. The interaction of lactoferrin with s-IgA could contribute to the accumulation of lactoferrin in sialoliths. In summary, these results suggest a new pathophysiological role for lactoferrin, in the formation of sialoliths.

Keywords

Sialolith, salivary stone, protein composition, lactoferrin, lysozyme

INTRODUCTION

Salivary stones or sialoliths are calcified structures which may occur, mainly unilaterally, in the salivary glands or their ducts. They can cause partial or total stagnation of the salivary flow and their presence is often associated with pain, swelling and infection of the salivary glands (Delli et al., 2014). Sialolithiasis is a relative common salivary gland disease occurring in approximately 0.1 to 1 percent of the population worldwide (Grases et al., 2003). Sialoliths occur most frequently in the submandibular (72-95%) and parotid (4-28%) glands and their ducts. In general, the etiology of sialoliths is not clear. The exact cause appears to be multifaceted and various hypotheses have been put forward. The formation seems to be related with factors such as hyposalivation, dehydration, and impaired crystalloid solubility (Avishai, Ben-Zvi, et al., 2021; Capaccio et al., 2007), agglomeration of sialomicroliths (Harrison et al., 2009) and anatomical variation of the excretory salivary ducts (Nagra et al., 2010). A decreased secretion rate and/or altered biochemical composition of saliva may also be a possible explanation for the formation of salivary stones (Kraaij et al., 2014). It has been reported that the salivary concentration of phytate and citrate, both crystallization inhibitors, and magnesium was decreased in sialolithiasis patients (Grases et al., 2003; Su et al., 2010). The saliva of patients suffering from salivary stones is more viscous and has a higher protein concentration compared to healthy individuals (Afanas'ev et al., 2003).

Histologically, a sialolith consists of a mineralized nucleus surrounded by various laminated layers of organic and inorganic compounds. Research has attempted to identify individual compounds of the sialolith. Hydroxyapatite, whitlockite and calcium phosphate are the major mineral components and are located at the outer layers of the sialolith (Kasaboğlu et al., 2004). Salivary stones also contain organic material like lipids, carbohydrates and proteins (Nolasco et al., 2013), the latter consisting approximately 5% of the dry weight of submandibular salivary stones (Slomiany et al., 1983). Amino acid analysis of sialolith proteins showed relatively high levels of alanine, leucine, glutamine, aspartic acid, valine and glycine (Harrill et al., 1959; Osuoji & Rowles, 1974). Using immunoblotting techniques, an unidentified, high-molecular weight glycoprotein was detected in solubilised submandibular sialoliths and lower molecular weight proteins, including statherin and acidic proline-rich proteins, were also identified (Proctor et al, 2005). Recently, using liquid chromatography-mass spectrometry Busso et al. (2020) detected between 116 and 419 unique proteins in salivary stones. Analysis of this study focused on the finding of homologies with proteins from bone, tooth and periosteal

tissue. Interestingly, it appeared that sialolith formation presented similarities with the hyperoxaluria that forms kidney stones. The glandular origin of the sialoliths studied, however, was not reported (Busso et al., 2020).

The aim of the current study was to explore the possible presence of salivary proteins involved in the formation of submandibular salivary stones, especially proteins involved in oral microbial defence and immunity, such as secretory-IgA, MUC7 and lysozyme (Amerongen & Veerman, 2002). Human defensin (HP3, HNP3 or DEF3), has been detected in the outer layer of the nucleus of salivary stones using MALDI-TOF (Hiraide & Nomura, 1980). This antimicrobial protein is secreted by epithelial cells of the excretory ducts of the salivary glands and by neutrophils. When neutrophils come into contact with calcium crystals, bacteria or when the pH is highly fluctuating, an inflammatory reaction occurs, called NET formation (Neutrophil Extracellular Trap). NETs promote adhesion of crystals and proteins, resulting in formation of macroscopic stones (Albar et al., 2014). As neutrophils also secrete lactoferrin, we investigated whether lactoferrin is present in salivary stones. Besides, systemic inflammatory markers i.e. C4, representing the complement system, and CRP, as suggested elsewhere (Avishai, Rabinovich, et al., 2021), were included in this analysis.

Materials and methods

Patients and Samples

Twenty submandibular salivary stones were obtained by endoscopic or trans-oral surgical removal at the departments of Oral and Maxillofacial Surgery. After stone removal, the sialoliths were rinsed with tap water or 0.9% saline solution, placed in a plastic container, and transferred to the laboratory. The stones were weighed using a precise scale (SartoriusGenius, Nieuwegein, The Netherlands), freeze dried overnight (Christ LT-105, Osterode am Harz, Germany) and weighed again. Subsequently, the salivary stones were stored at -20°C until biochemical analysis.

The salivary stones were homogenized with an aluminium pestle, and 10mg pulverized salivary stone was mixed with 200µL 1x SDS reducing sample buffer (Thermo Fisher Scientific, Waltham, Ma, USA) and boiled for five minutes. The suspension was then clarified by centrifugation for five minutes at 4000 rpm, 30*g* (Eppendorf centrifuge 5810, Hamburg, Germany). The supernatant was used for gel electrophoresis.

Gel electrophoresis

SDS-PAGE was performed on NuPAGE 4–12% BisTris gels (Life Technologies, Carlsbad, Ca, USA) under reducing conditions. Samples were loaded on the gel and run for 35 minutes at 200V (Xcell4 Sure LockTM Midi-cell, Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's protocol. Novex sharp pre-stained proteins standards (Thermo Fisher Scientific, Waltham, USA) were used as molecular mass markers. The gels were then incubated with Coomassie Brilliant Blue (R-250) stain for three hours at room temperature, followed by overnight de-staining in 10% acetic acid.

Western Blotting

Proteins extracted from salivary stones were separated on 4-12% SDS PAGE gels and transferred to nitrocellulose membranes by semi-dry blotting (iBlot Invitrogen, Thermo Fisher Scientific) according to manufacturer's protocol. Nitrocellulose membranes were incubated for one hour with various antisera against salivary proteins: rabbit polyclonal antibody to human lactoferrin (L-3262) (1:500) (Sigma Chemical Co., St. Louis, Mo, USA), mouse monoclonal antibody to amylase (sc-166349) (1:1000) (Santa Cruz), rabbit polyclonal antibody to MUC7 (2A4) (1:500) (ACTA Oral biochemistry), rabbit polyclonal antibody to

human lysozyme (A099) (1:500) (Dako, Glostrup, Denmark), rabbit polyclonal antibody to human s-IgA (A0187) (1:500) (Dako, Glostrup, Denmark), mouse monoclonal antibody to human CRP (C1688) (1:1000) (Sigma-Aldrich) and biotinylated mouse monoclonal antibody to human complement C4 (1:1000) (Sanquin, Amsterdam, Netherlands). The salivary protein antibodies were detected with the recommended labelled secondary antibody conjugates (1:1000): goat anti rabbit AP (alkaline phosphatase), rabbit anti mice AP (Dako) and streptavidine AP (Caltag Laboratories, Burlingame, United States). The membranes were stained with Sigmafast BCIP/NBT (Sigma Aldrich).

Protein extraction

Per 50mg dry weight of pulverized salivary stone, 1 mL 1:1 methanol-chloroform mixture was added. The mixtures were 30 minutes exposed to a 20kHz digital sonifier S-250A (Branson Ultrasonic Co., Danbury, USA). The suspensions were placed overnight on a rotating wheel at 10 rpm (Stuart rotator SB3, Staffordshire, UK). Next, 1 mL of distilled water was added, followed by sonication for 45 seconds until a cloudy suspension was obtained. The suspensions were placed for 72h at room temperature on a rotating wheel at 10 rpm. Subsequently, the suspensions were centrifuged for 10 minutes, 4000 rpm, 30*g* (Eppendorf centrifuge 5810, Hamburg, Germany).

The supernatants were transferred to a new Eppendorf vial, frozen in liquid nitrogen, lyophilized (Christ LT-105) and stored at -20ºC. To extract any residual material, the pellets were dissolved in 0.5 mL 0.1 M Na_2CO_3 (pH 9.6) coating buffer, homogenized on a vortex mixer (full speed, 1 minute) and centrifuged for 10 minutes at 4000rpm, 30*g* (Eppendorf centrifuge 5810, Hamburg, Germany). The resulting supernatants were added to the lyophilized supernatant from the chloroform-methanol extraction step. Total protein content in the thus obtained solution was measured in 96-well polystyrene microplates using the BCA protein Assay Kit according to the instructions of the manufacturer (Thermo Scientific). Optical readouts for the BCA assay and for all ELISA's performed in this study were obtained using a Multiscan FC microplate photometer (Thermo Scientific).

ELISA

All ELISA's were performed in 96-well, high-binding polystyrene microplates (Greiner Bio-One, Kremsmünster, Austria). 25µL of the total supernatants obtained during the protein extraction step were added to a microplate well and 175µL coating buffer was added.

Then, two-fold serial dilutions of each supernatant were prepared in coating buffer, and incubated overnight at 4ºC. Protein levels were determined as previously described (Bolscher et al., 1999; Prodan et al., 2015). The following antibodies have been used: rabbit polyclonal antibody to lactoferrin (L-3262) (1:1000), rabbit polyclonal antibody to lysozyme (A099) (1:300), rabbit polyclonal antibody to s-IgA (A0187) (1:1000) and rabbit polyclonal antibody to PRP (1:1000) (Dako, Glostrup).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 28.0 (IBM Inc, Armonk, USA), using Spearman's rank order coefficient. P values < 0.05 were considered statistically significant.

Ethical approval

This study was approved by the Medical Ethical Committee of the Amsterdam UMC, location VUmc (protocol number 2012/127) and informed consent was obtained from all patients. During the course of this study all guidelines and protocols of the Declaration of Helsinki were followed.

Results

There were twenty submandibular stones which were obtained from eleven males and nine females, with an average age of 53 years (median 50.5 years). (Table 1) The sialoliths showed a wide range in weight (61.52mg – 1113.61mg) and total protein concentration ranged from 157 – 866 (mean 468 ± 127 µg/mL). The negative correlation between the protein concentration in submandibular salivary stones and the dry weight of the sialoliths almost reached statistical significance (Spearman's rangorder correlation r=-0.456, p=0.066). This suggests that salivary stones with dry weight up to approximately 250mg contain relatively more extractable proteins than salivary stones with higher dry weight. (Figure 1). In this study, 55% of the stones had a dry weight between 51.36 and 250 mg, and 45% of the stones had a larger dry weight, up to 842mg. Using gel electrophoresis, the protein profiles of the twenty different submandibular salivary stones showed individual variations (Figure 2). Using a combination of SDS-PAGE and Western blotting, several specific proteins could be identified. Alpha-amylase was detectable in 20 of the 20 salivary stones (100%), lysozyme in 19 of the 20 stones (95%), lactoferrin in 17

of the 20 stones (85%), s-IgA in 15 of the 20 stones (75%), MUC7 in 12 of the 20 stones (60%), complement C4 in 12 of the 20 stones (60%) and C-reactive protein in 7 of the 20 stones (35%) (Figure 3 and attachments figures 1-7).

	Total weight (mg)	Dry weight (mg)	Age (years)	Gender	Side
1	185.70	136.65	73	M	L
2	525.48	472.75	43	M	L
3	439.77	291.46	31	M	R
4	433.18	369.72	43	M	R
5	597.98	376.72	71	F	R
6	301.78	253.11	49	M	L
7	117.27	88.52	51	M	R
8	146.17	109.91	79	F	R
9	383.59	328.61	56	F	L
10	263.01	206.90	46	M	L
11	814.55	674.57	72	F	L
12	256.12	239.45	36	M	L
13	207.91	164.64	40	M	L
14	202.59	164.19	62	F	R
15	61.52	51.36	39	F	R
16	260.14	237.48	55	F	R
17	391.51	300.00	50	M	R
18	1113.61	841.99	26	M	R
19	147.01	117.49	61	F	L
20	88.14	78.29	66	F	L
Average	346.85	275.19	52.45		
SD	260.89	201.35	14.92		
Range	61.52 – 1113.61	51.36 – 841.99	26 - 79		

Table 1 – Characteristics of patients and their salivary stones (n=20)

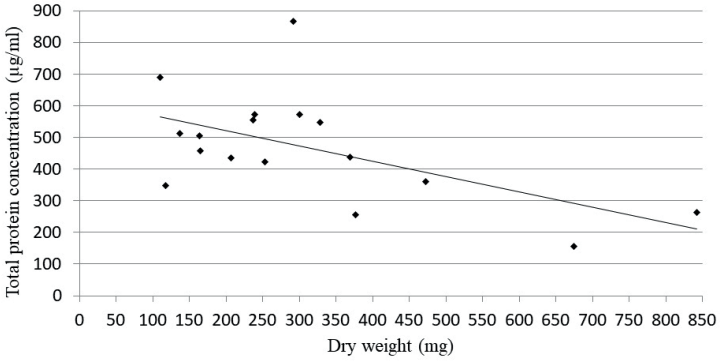

Figure 1- Relationship between dry weight and total protein concentration of submandibular sialoliths.

Using ELISA, the presence of lactoferrin (7.38±10.44 µg/mL), s-IgA ($4.07*10^{-3}$±$3.77*10^{-3}$ µg/mL) and lysozyme (9.86±7.72 µg/mL) in sialoliths was confirmed (Table 2). No detectable levels of PRP were found. The age of the patient did not correlate significantly with the concentration lactoferrin, lysozyme, sIgA or total protein. We also did not see any significant differences in concentration lactoferrin, lysozyme, sIgA or total protein between sialoliths from women and men. The concentration lysozyme and sIgA did not correlate with the total protein concentration. However, the lactoferrin concentration in sialoliths showed a significant, positive correlation with the total protein concentration (r=0,514, p=0.035).

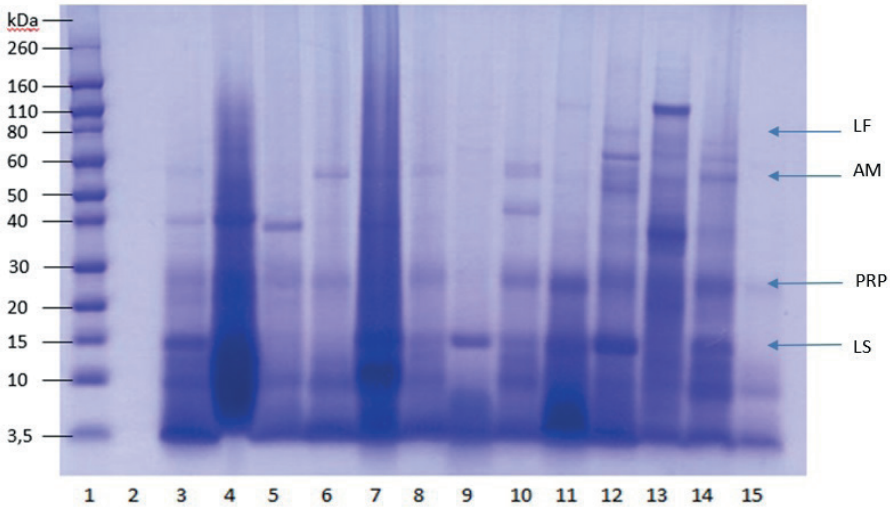

Figure 2 - SDS PAGE analysis of the protein composition of submandibular salivary stones from 13 different individuals (lane 3-15), shows the large variety of proteins and protein levels in salivary stones. Lane 1: pre-stained molecular weight markers.

Indicated are the molecular weights of Lysozym (LS, 15kDa), a-amylase (AM, 55kDa), lactoferrin (LF, 80kDa) and proline rich proteins (PRP, 20-30kDa), as reported by (Becerra et al., 2003) and (Van Nieuw Amerongen et al., 2004).

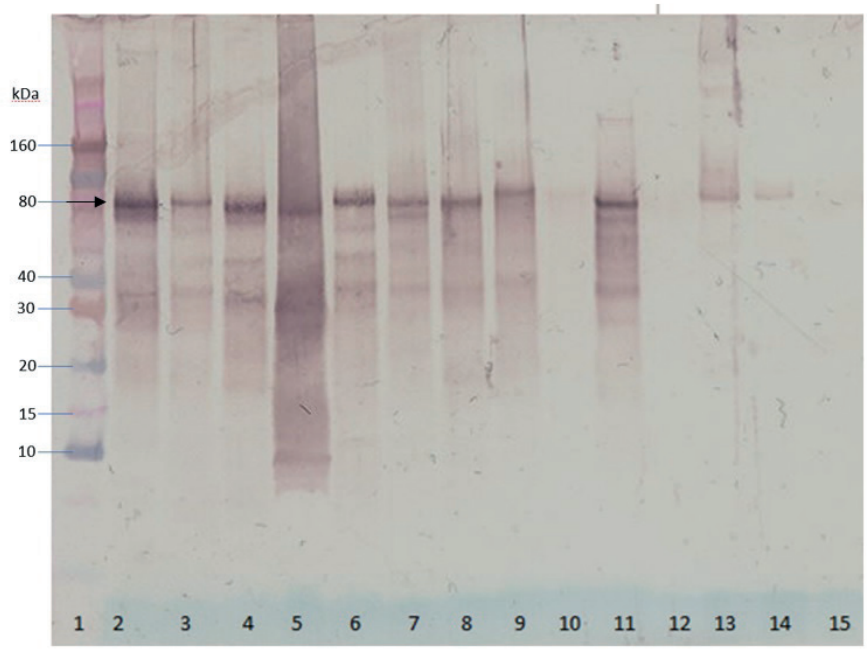

Figure 3 – Immunoblot with antibody against lactoferrin (indicated by arrow) of proteins extracted from submandibular sialoliths of 13 different individuals (lane 2-14). Lane 1: pre-stained molecular weight markers.

	Mean concentration	**SD**	**Range**
Lactoferrin (µg/mL)	7.38	10.44	0.02 – 80.0
sIgA (µg/mL)	$4.07*10^{-3}$	$3.77*10^{-3}$	$0.49*10^{-3}$ – $25.38*10^{-3}$
Lysozyme (µg/mL)	9.86	7.72	0.18 – 22.63

Table 2 – Concentrations of several proteins in submandibular sialoliths, determined with ELISA (n=17)

Discussion

The study population of this pilot study comprised eleven men and nine women. This is in line with recent studies reporting an almost equal distribution of salivary stones between men and women (Kraaij et al, 2014). In the sialoliths of the subjects, the presence of proteins was established which is in agreement with other studies. (Busso et al., 2020; Isacsson & Hammarström, 1983). The almost significant negative correlation between the total protein concentration and dry weight of the submandibular salivary stones indicates that smaller salivary stones (≤250mg) contain relatively more proteins compared to larger stones. This can be explained by the fact that proteins are only present in the core of the salivary stone and layered growth around the nucleus is mainly caused by inorganic materials. This is in agreement with the study of (Szalma et al., 2013), which showed that proteins are mainly present in the core of the sialoliths.

In the present study we frequently identified lysozyme (95%), lactoferrin (85%) and s-IgA (75%) in the submandibular sialoliths. In a vast majority of the 20 stones examined, these three proteins were simultaneously present. The concomitant presence of lactoferrin, lysozyme and s-IgA in sialoliths might be explained by the fact that both lactoferrin and lysozyme have been shown to bind to s-IgA. (Kugler et al., 1996) S-IgA enhances the antimicrobial properties of lactoferrin (Sharma et al., 2017) And lysozyme is a protein that lyses bacteria and may work synergistically with lactoferrin and sIgA in antibacterial functions (Garofalo & Goldman, 1999). Together, these proteins may reduce the risk of bacterial overgrowth of a developing submandibular sialolith.

Lysozyme, a protein which occurs in relatively low concentrations in unstimulated submandibular saliva (6-15 µg/mL) (Yeh et al., 1997), could be detected in almost all salivary stones. A possible explanation is that lysozyme binds well to calcium phosphate so that it will accumulate in salivary stones (Kraaij et al., 2014; Zhu et al., 2007). Lysozyme is not only secreted by the salivary glands, but also secreted by inflammatory cells, especially neutrophils (Fábián et al., 2012). It is possible that lysozyme in sialoliths does not originate from saliva but from neutrophil infiltration as a result of recurrent subclinical salivary gland inflammation due to the sialolith. This explanation could also apply to the presence of lactoferrin in sialoliths. Lactoferrin occurs in low concentrations in unstimulated whole saliva (8.96µg/mL) (Rosa et al., 2021), but is an abundant neutrophil-derived protein, that can be rapidly mobilized to aid the host defense response at sites of infection throughout the human body. It protects epithelial cells against microbial infection, presumably by

binding to surface bacterial proteins and blocking their adhesion to host cells (Ward et al., 2005).

Unfortunately, the results of SDS-PAGE and the ELISA assays of this pilot study are not completely unambiguous. Despite the fact that the amounts of lactoferrin and lysozyme detected by ELISA in sialolith extracts were almost comparable (Table 2), the band migrating in Figure 2 at 15 kDa (lysozyme) is relatively strong compared to the bands migrating around 70 kDa (lactoferrin) (Becerra et al., 2003). This raises the question whether the high ELISA readings with the polyclonal lactoferrin antiserum are due to other constituents in the sialolith extracts. This hypothesis is supported by considerable reactivity in other regions of the sample in Figure 3. That potentially could account for some of the higher ELISA readings. On the other hand, the commercially available polyclonal antibody against lactoferrin used in the present study clearly recognizes human lactoferrin (Hu et al, 2015). Inclusion of a commercially available lactoferrin preparation in control lanes of the SDS-PAGE gel to see whether it also contains reactivity elsewhere would constitute a valuable control in future studies.

Other salivary proteins were identified as well, including amylase, MUC7 and PRP's. It has been reported that saliva of patients suffering from salivary stones is more viscous and contains a higher total protein concentration (Afanas'ev et al., 2003). Therefore, it would be interesting for a follow-up study to compare the salivary protein composition of patients with sialoliths and healthy subjects, to explore whether they differ in protein concentration of the proteins identified in sialoliths in the present study.

Despite the washing protocol immediately after removal, using water or 0.9% sialine, sialoliths may have been contaminated with blood. As a result, some of the serum proteins detected may have derived from the blood rather than from the sialolith. This could apply, for example, for the inflammatory proteins complement C4 and C-reactive protein (CRP) which were identified in 60 and 25% of the submandibular sialoliths, respectively. However, the most abundant proteins in the sialoliths usually have very low concentrations in blood and thus it seems unlikely that contamination with blood could have a significant effect on the results of the current study. Future research on the location of the different proteins within sialoliths, the interactions between these proteins and the presence and possible role of lactoperoxidase and inflammatory parameters such as interleukin 6 and neutrophils is needed. This information may contribute to the understanding of the pathogenesis of sialoliths.

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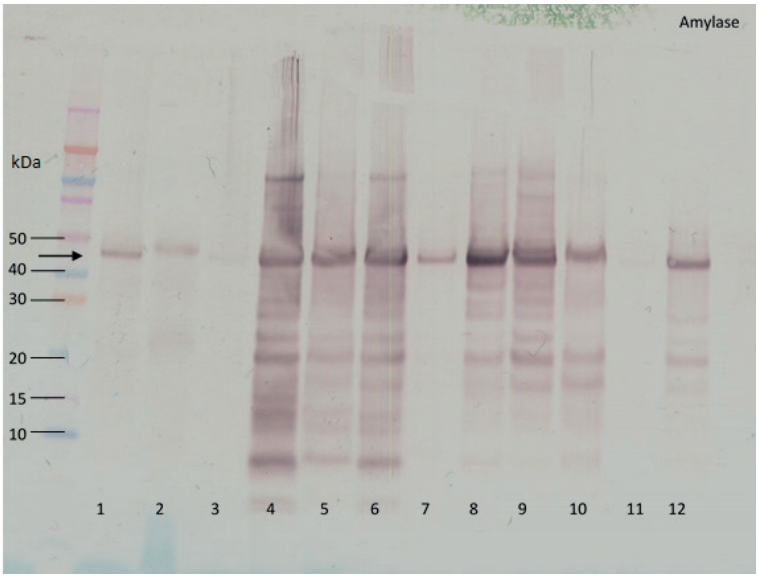
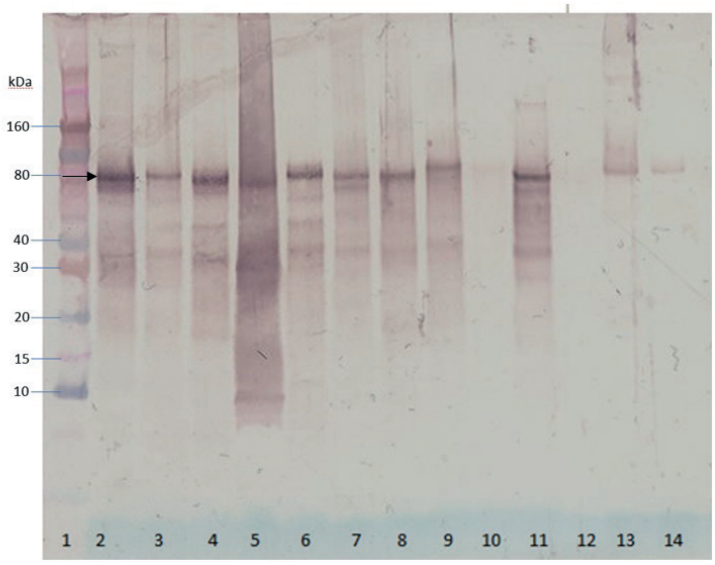
Attachment figure 1: Western Blot – Amylase



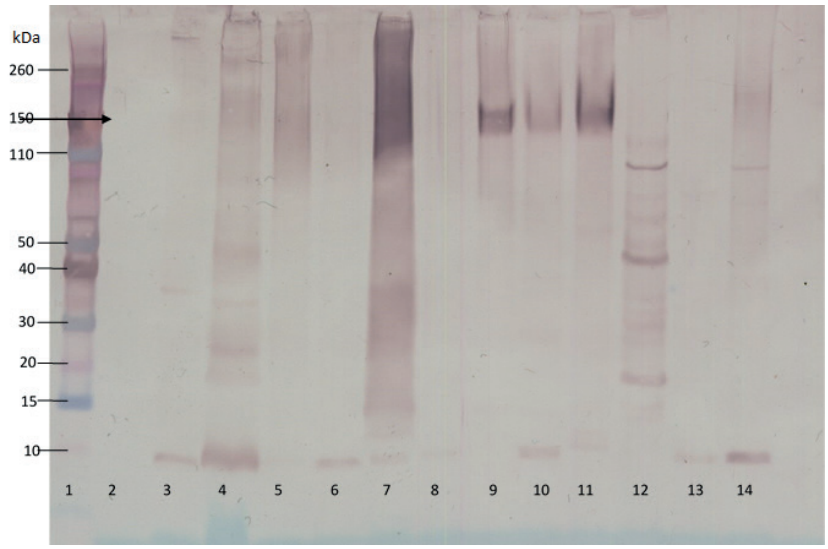
Immunoblot with antibody against amylase (indicated by arrow) of proteins extracted from submandibular sialoliths of 12 different individuals (lane 1-12). Lane 0: pre-stained molecular weight markers.

Attachment figure 2: Western Blot – Lactoferrin



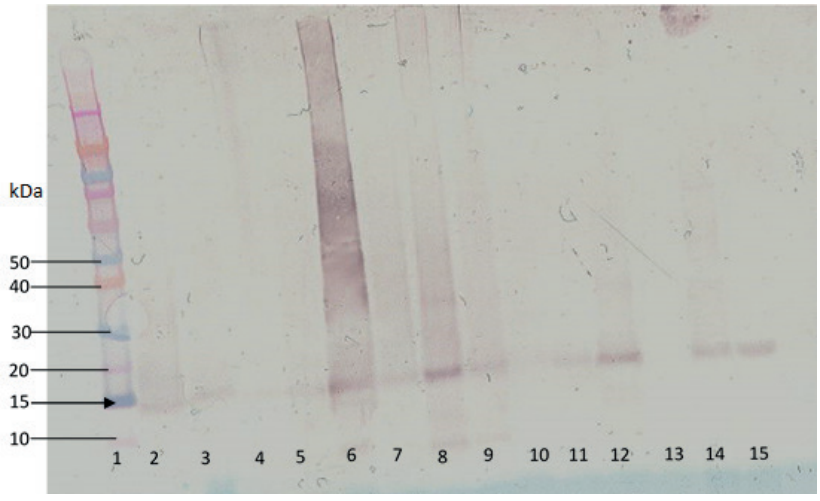
Immunoblot with antibody against lactoferrin (indicated by arrow) of proteins extracted from submandibular sialoliths of 13 different individuals (lane 2-14). Lane 1: pre-stained molecular weight markers.

Attachment figure 3: Western Blot – MUC7



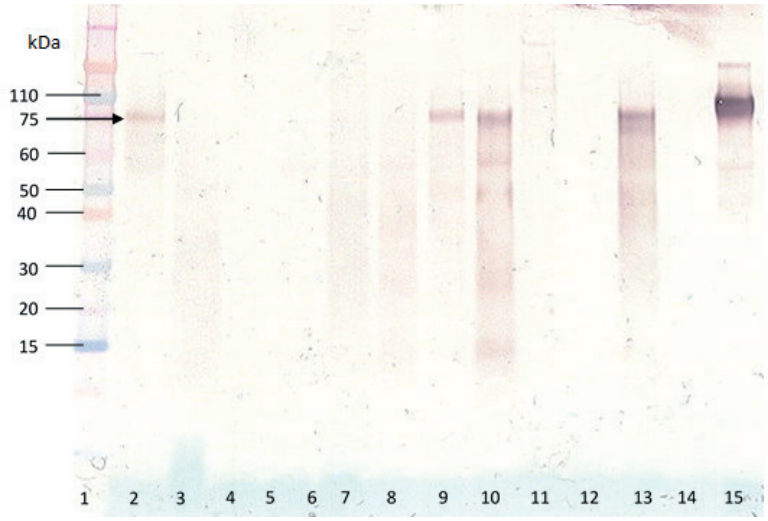
Immunoblot with antibody against MUC7 (indicated by arrow) of proteins extracted from submandibular sialoliths of 12 different individuals (lane 3-14). Lane 1: pre-stained molecular weight markers.

Attachment figure 4: Western Blot – Lysozyme



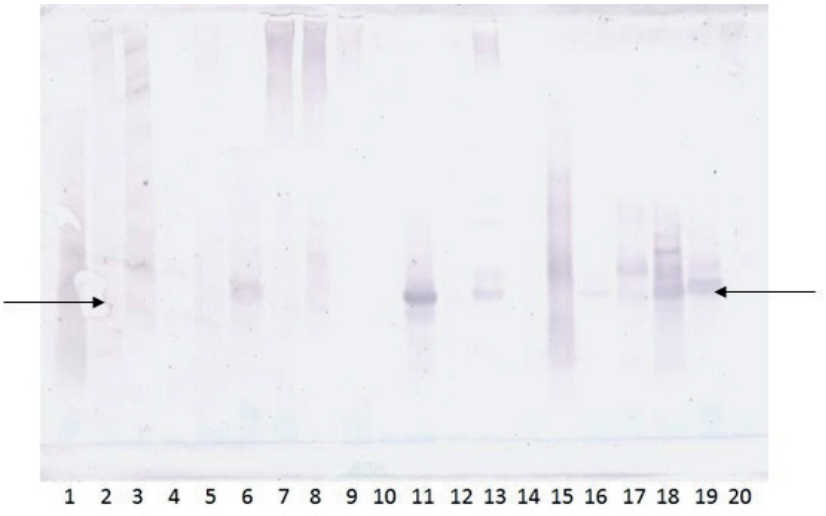
Immunoblot with antibody against lysozyme (indicated by arrow) of proteins extracted from submandibular sialoliths of 14 different individuals (lane 2-15). Lane 1: pre-stained molecular weight markers.

Attachment figure 5: Western Blot – S-IgA



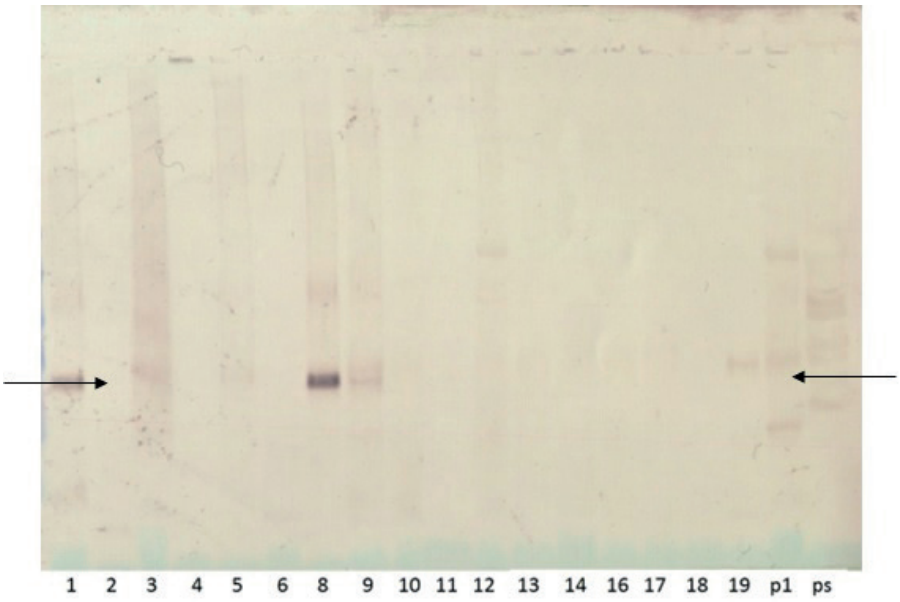
Immunoblot with antibody against s-IgA (indicated by arrow) of proteins extracted from submandibular sialoliths of 13 different individuals (lane 2-15). Lane 1: pre-stained molecular weight markers.

Attachment figure 6: Western Blot – C4

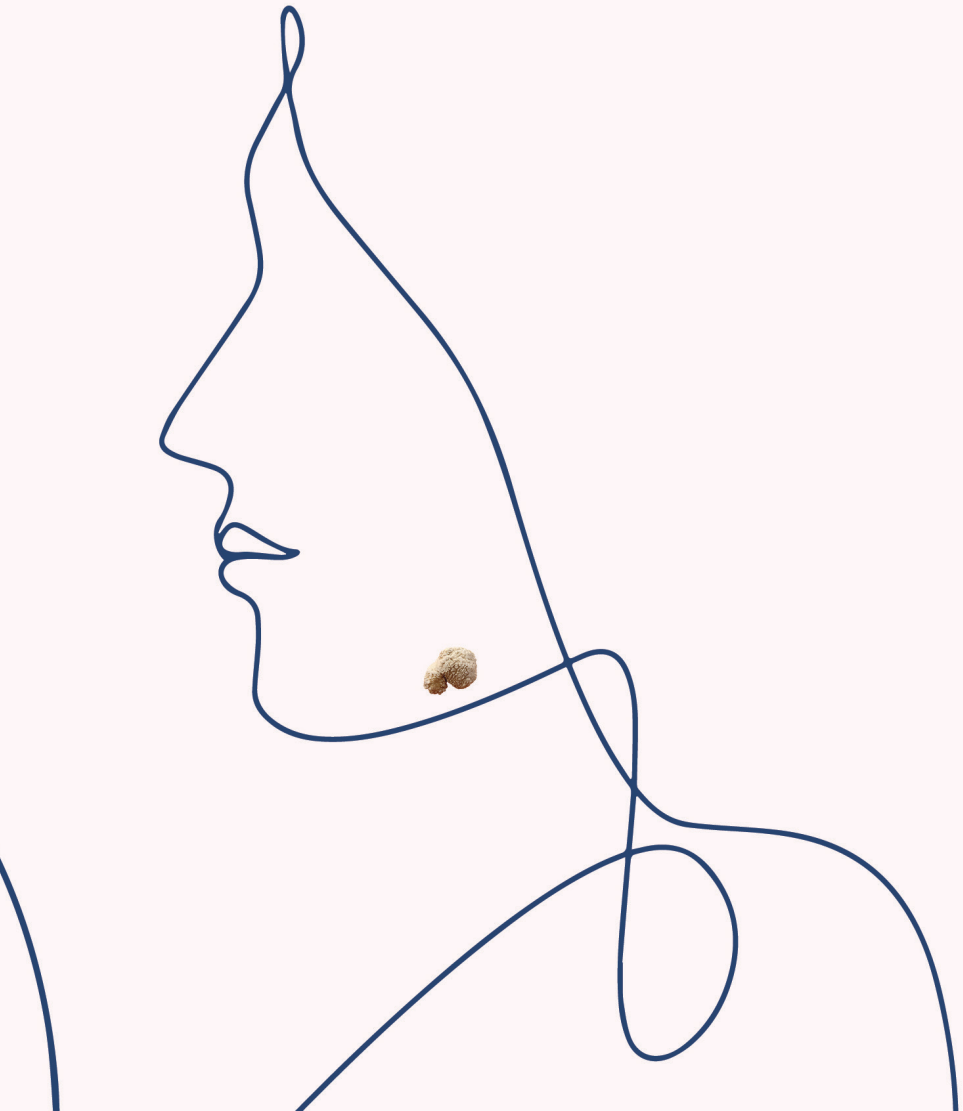


Immunoblot with antibody against C4 (indicated by arrow) of proteins extracted from submandibular sialoliths of 18 different individuals (lane 1-19).

Attachment figure 7: Western Blot – CRP



Immunoblot with antibody against CRP (indicated by arrow) of proteins extracted from submandibular sialoliths of 18 different individuals (lane 1-19, p1, ps).



CHAPTER 7

General discussion

GENERAL DISCUSSION

Lithiasis is defined as the pathological accumulation of calcified structures in organs, which may lead to obstruction and often to inflammation. Typical examples include, among others, nephrolithiasis, prostatic lithiasis, cholelithiasis, venous stones, tonsilloliths and sialolithiasis. Typical clinical symptoms of lithiasis comprise pain, swelling and recurrent infections (1,2). The annual incidence and prevalence of nephrolithiasis in The Netherlands are respectively 3 per 1000 and 8 per 1000 patients (3). This corresponds to the annual incidence of nephrolithiasis in the (Western) industrialized world which is considered to be 2 cases per 1000 (4). The cumulative 10-year incidence of asymptomatic cholelithiasis is 6.7 per 100 and the prevalence of asymptomatic cholelithiasis ranges from 13 to 22 per 100 and increases with age (5,6,7).

The incidence of sialolithiasis is relatively low with an estimated incidence in both England and Switzerland of 1 to 10.000-30.000 per year (8,9,10). The first observation of sialolithiasis was made by Peterson in 1946 (11). In the reported case, clumps of hydroxyapatite were found in both the parotid gland and the submandibular gland. From that moment on salivary stones gained interest among clinicians and researchers. Since then, over 2100 papers have been published in English language, medical scientific journals.

Inorganic composition

Salivary stones are biominerals, formed in a specific microenvironment, and mainly consist of carbonates and phosphates. The composition of materials in stones is more or less comparable with the content of human hard tissues such as teeth and bones. The composition and structure of salivary stones reflects specific characteristics of their formation process and respective location. In line, in **Chapters 2 and 5**, the inorganic composition of salivary stones was described. It was found that the majority of submandibular salivary stones consist of hydroxyapatite ($Ca_5(PO_4)_3OH$, which is also the main mineral of the tooth enamel, whitlockite ($Ca_3(PO_4)_2$), octacalciumphosphate ($Ca_8H_2(PO_4)_6.5H_2O$) and brushite ($CaHPO_4.2H_2O$), which is consistent with previous research (9,12,13).

Hydroxyapatite formation of the teeth is roughly based on three stages: induction, crystallization and maturation. Although the exact formation mechanism of submandibular stones has yet to be elucidated, we feel it tempting to hypothesize that stone formation

might be prevented or disrupted by inhibition of the induction and/or the crystallization stage of hydroxyapatite formation. Saliva from people with a salivary stone in the ductal system of the submandibular gland, contains significantly more Ca^{2+} than the saliva of healthy individuals. This suggests that reducing consumption of Ca^{2+} rich foods, such as dairy products (milk, yogurt, cheese) as well as green vegetables, nuts and legumes, possibly reduce the risk of stone formation (14). In addition, saliva of patients with increased risk of sialolithiasis contain low levels of crystallization inhibitors such as citrate, phytate and magnesium (1,14).

Chapter 3 described the accuracy of a Cone Beam Computed Tomography (CBCT) scan for diagnosing the size and form of salivary stones. It was found that stones are generally 19.7% smaller, measured *in vivo* on micro-CT, compared to when measured preoperatively on the CBCT scan. Nevertheless, CBCT is the first-choice imaging technique when the signs and symptoms of a patient indicate the possible presence of a salivary stone. This is because of the low costs, usual immediate availability and limited radiation exposure. As compared to a spiral CT, ultra sound is a good alternative option but with this technique stones smaller than 3mm are missed because they do not produce a dorsal acoustic shadow or are not hyperechoic with respect to surrounding structures. The lack of an acoustic dorsal shadow may depend not only on the size but also on the biochemical composition of the calculi due to the lack of an adequate amount of Ca^{2+}.

Organic composition

Sialoliths are mainly composed of inorganic compounds but also contain organic compounds such as lipids and proteins. Protein contributes approximately 5% to the stone weight of submandibular stones. In **Chapter 6**, the possible correlation between protein concentration and dry weight of the stones was described. A negative correlation was found between both variables, suggesting that protein was mainly found in the core of the stone and that the peripheral layers contained little to no protein. However, in stones analyzed using scanning electron microscope (SEM) and x-ray diffraction analysis, organic constituents were found mainly in the outer shell of the stone (12,15).

In **Chapter 6**, 20 submandibular salivary stones were tested for saliva-specific proteins by gel electrophoresis, western blot and ELISA. The mean dry weight of the 20 stones used is 275.19 mg, (range 51.36 – 841.99 mg) which represents a large spread in weight of the stones. Sabot et al (2012) (16) found a comparable distribution of weight in their

study including 74 stones. Because of the limited material available per stone, it was not possible with the analytical methods used, to test each stone for all conceivable proteins, for example for human salivary proteins as well as proteins derived from bacteria. Yet, we did manage to demonstrate that in addition to salivary proteins (Lysozyme, sIgA, α-amylase, lactoferrin and MUC7), inflammatory proteins (C4 and CRP) are also present in the stones. Sabot et al (2012) also found salivary and inflammatory proteins using mid-infrared spectrometry and showed that 75% of their stones contain protein. Mucins represented the major protein component and albumin was found in 10% of all the specimens.

Recent, Busso et al (2020) (17) described how they used proteomics in combination with liquid chromatography mass spectrometry (LCMS) to explore the protein composition of 29 submandibular salivary stones. Using this technique, it was possible to identify a large number (824) of proteins with a limited amount of stone sample. The downside of this technique is that it produces a very large data set and it is difficult to obtain relevant results from it. Czaplewska et al (2021) (18) also used proteomics to analyze submandibular salivary stones. They modified the protocol of Busso et al (2020), so that protein extraction from stones was optimized. These preliminary studies show that salivary stones contain a significant number of bacterial proteins in addition to human proteins. These are derived from natural bacteria from the flora of the salivary glands and pathogenic bacteria, deposited in the salivary stone along with human proteins and inorganic material.

Limitations of our studies

The data sampling related to this thesis took place at different departments of Oral and Maxillofacial Surgery in the Netherlands. This can be seen as an advantage, for instance, as it corrects for local differences in water hardness and possible unknown regional factors. The study by Sherman and McGurk (2020) (19) described that the incidence of salivary stones and sialadenitis have similar anomalous patterns plotted against different regions in the United Kingdom. They were not able to identify regional factors such as water hardness that could have played a role.

The multicenter model used in our studies, however, also has limitations. The stones were collected from different hospitals distributed across the Netherlands and despite pre-established protocols, transport to the laboratory often did not take place on the same

day, and in the intervening time (between removal and storage at the ACTA laboratory at -20°C) the stones were not always stored in the same way, whether at room temperature, in the refrigerator (-4°C) or at -20°C.

Salivary stones used for investigation in this thesis were mainly from the submandibular gland (n=355; 92%) and some of the parotid gland (n=29; 8%). The distribution of stones over both salivary glands are consistent with the literature (9,20). A considerable part of these parotid stones' volume and mass were too small to be processed for inorganic and organic analysis.

In **Chapter 5** it was described that the composition of salivary stones in older patients was different compared to younger patients (<37years). This may be related to an age-dependent growth of the stone. A stone "grows" about 1-1.5 mm in diameter each year (21). Stones originating from the parotid ductal system and gland are generally smaller than those originating from the submandibular ductal system and gland. When a parotid stone has enough volume so that it can be analyzed, it will be a stone either harvested from an older patient and or a symptomless stone that has been present for several years. Therefore, the composition of parotid stones could have been biased by the fact that they can only be analyzed when they have enough mass and volume.

Future plans

Scientific research on various aspects of salivary stones is very broad, with respect to their origin, composition, diagnosis and treatment options. Accordingly, suggestions for further research includes various aspects.

1) In this thesis, a pilot study was described where a start was made to investigate protein composition in submandibular salivary stones (**Chapter 6**). In future studies, the protein composition of parotid stones should also be examined, as the composition of parotid saliva differs from submandibular saliva (serous vs mixed mucous-serous). The expected difference in composition, both organic and inorganic, of submandibular and parotid stones and the fact that parotid stones are much less common compared to submandibular stones, may provide new insights regarding the difference in formation of parotid and submandibular salivary stones.

2) Research on saliva of patients suffering from sialolithiasis would be a good addition to current research. After stone removal, the treated salivary gland is expected to recover

to a normally functioning gland (22). It has been reported that saliva of patients suffering from salivary stones is more viscous and contains a higher total protein concentration (23). Does the composition of saliva change as the gland recovers? And if so, what does this say about the origin of the stone?

3) The dental curriculum at universities in Europe is not uniform. This is due to different cultural backgrounds, history and educational philosophies. This ultimately leads to enormous diversity and quality of dental education (24). In general, all universities pay, in a greater or lesser extent, attention to saliva-related topics in their education. However, it is not clear whether education specifically on salivary stones is provided (25). When oral healthcare professionals have received less education regarding specific signs, symptoms, and clinical and radiological topics, they might be less likely to recognize a disturbed salivary flow of caused by salivary stones and related salivary gland pathology. Thorough education about various clinical aspects of saliva and salivary gland pathology could ensure that sialolithiasis related diseases may be well diagnosed. Early diagnoses might result in less damage to the affected salivary gland and fewer symptoms for the patient.

4) Artificial intelligence (AI) aims to mimic human cognitive functions and is progressing rapidly. An AI system can help to reduce diagnostic and therapeutic errors that are inevitable in the human clinical practice (26). In dentistry, these algorithms are expected to improve the accuracy and efficacy of dental diagnosis, provide visualized anatomic guidance for treatment, simulate and evaluate prospective results, and project the occurrence and prognosis of oral diseases. (27) For example in Oral and Maxillofacial Surgery, AI can be used for diagnosing cysts and tumors, salivary gland abnormalities and anatomical guidance. Recently, a deep learning/ AI model for diagnosing salivary stones in both the parotid and submandibular salivary glands has been investigated (27). This would be an interesting and efficient addition for diagnostics and treatment planning.

This thesis presents the recent literature concerning salivary stones in the broadest context of the subject. In addition, risk factors of developing a salivary stone are described and a start has been made in identifying the proteins in salivary stones. As always with scientific research, specific additional research is needed, for example to further investigate the exact pathophysiology of salivary stones. New research could also focus on the effectiveness of current and new diagnostic and treatment modalities. In the future, hopefully this information could contribute to prevention in patients at increased risk of stone formation. Preferably, all knowledge about saliva and salivary stones will be made (digitally) available to clinicians and these topics will have a permanent place in dental curricula at universities.

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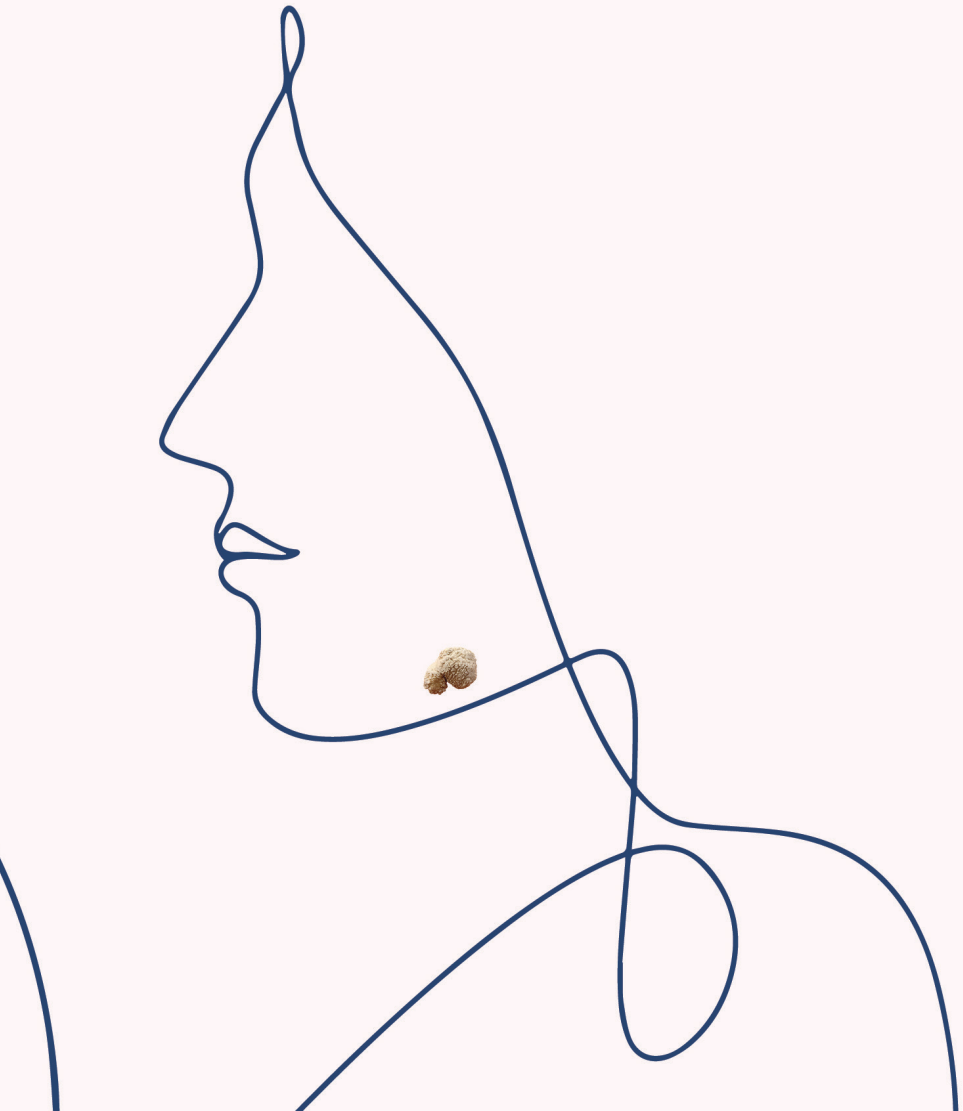

SUMMARY

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SUMMARY

Salivary stones or sialoliths are hardened, stone-like calcifications that develop primarily in the drainage ducts of a salivary gland and less frequently in the gland itself. The submandibular salivary gland is most frequently affected (84%) and less often the parotid salivary gland (13%). In both salivary glands, salivary stones are mostly located in the main excretory duct and less frequently in the small ducts and within the gland. Salivary stones occur incidentically in the sublingual salivary gland and accessory salivary glands.

The presence of a salivary stone can lead to the partial or complete obstruction of the excretory duct. As a result, symptoms such as recurrent, mealtime-related swelling and pain of the affected salivary gland, may occur. After several episodes of these mealtime-related symptoms, the swelling and pain are more or less continuously present. The severity of symptoms can vary and is determined by the localization and size of the salivary stone. A salivary stone may be symptomless and found as an incidental finding on an x-ray of the jaw.

The aetiology of salivary stones is unclear. Over the years, several hypotheses have been put forward that have attempted to explain the etiology and pathophysiology of salivary stone formation such as clumping of mucus plugs to form a large stone-like calcification. The higher incidence of salivary stones in the submandibular gland may be due to the length, winding and upward course of the main duct of the submandibular salivary gland (ductus Whartoni) and the higher viscosity of saliva from the glandula submandibularis compared to saliva from the glandula parotidea.

The research described in this thesis mainly focused on submandibular salivary stones with special attention to possible factors that may be involved in salivary stone formation, the radiological and clinical difference in size and volume of a salivary stone and the biochemical composition of salivary stones.

Chapter 2 reviews the knowledge on the aetiology, symptoms, biochemical composition and treatment of salivary stones based on current scientific literature. It was found that when salivary production increases, for example when smelling or thinking of food, this will cause a rise in intraglandular pressure in the salivary gland, resulting in an increase of pain and swelling of the affected gland.

Salivary stones consist of an amorphous mineralized core surrounded by concentric laminated layers of organic (collagen, proteins, amino acids and carbohydrates) and inorganic substances (hydroxyapatite, carbonateapatite, whitlockite and brushite).

Successful removal of salivary stones depends on preoperative information about the exact location, size and shape of the stones. The accuracy of a preoperative Cone Beam Computed Tomography (CBCT) scan in determining the volume of a salivary stone has been examined in **Chapter 3**. This study showed that when CBCT scans are used as a diagnostic tool in the detection of submandibular salivary stones, the stones are actually a fraction smaller than determined by radiological examination. This is of clinical importance since cut-off values for stone size are used in choosing the type of treatment for stone removal.

Little is known about the relationship between lifestyle factors and the presence of salivary stones. The possible relationship between the presence of salivary stones and systemic diseases, use of medication, smoking and alcohol consumption has been examined in **Chapter 4**. This showed that patients with salivary stones used significantly more antibiotics than the control group. No association was found between the occurrence of salivary stones and some common systemic diseases such as hypertension, diabetes mellitus and rheumatoid arthritis. Smoking and alcohol consumption played no or only a limited role in the presence of salivary stones.

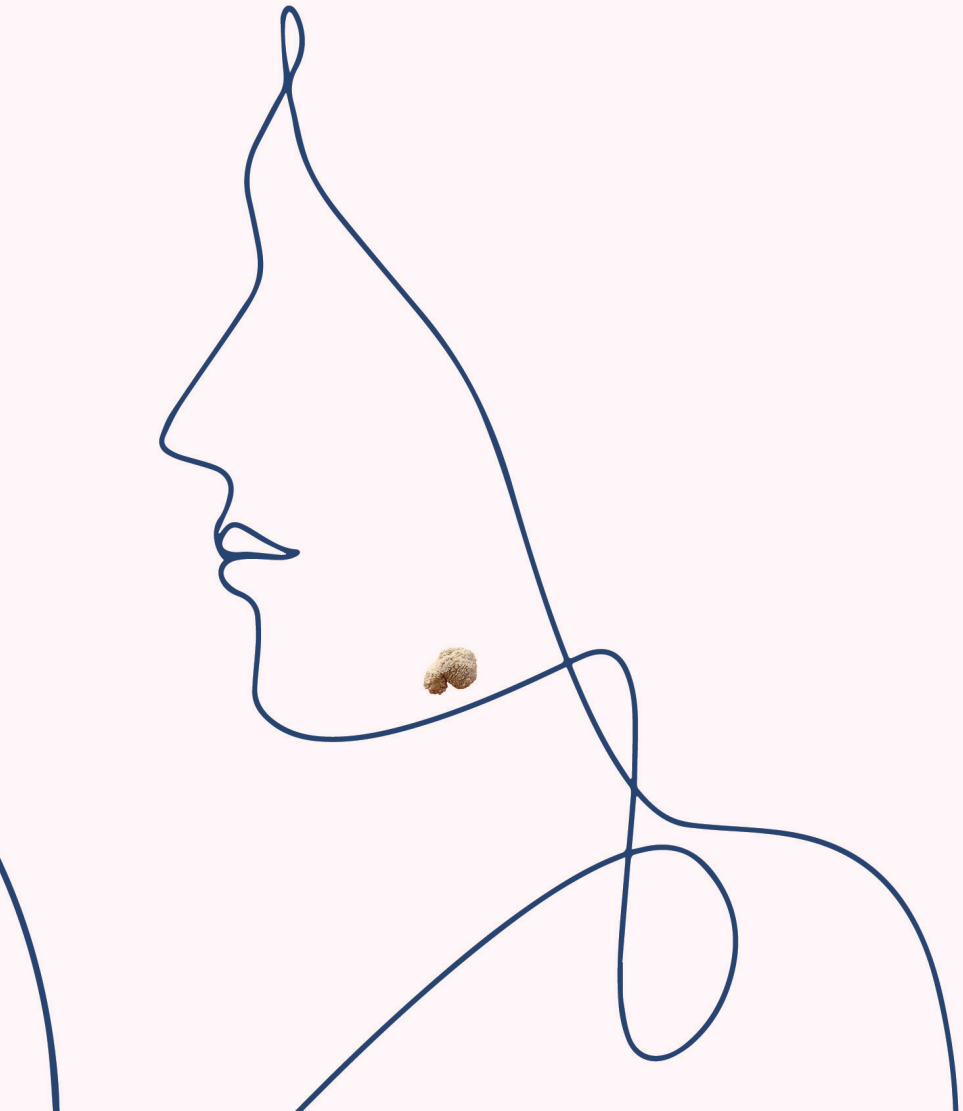
In **Chapter 5**, the biochemical, inorganic composition of submandibular and parotid salivary stones was determined and the possible relationship between stone specific characteristics and patient related characteristics investigated. Salivary stones were found to be composed primarily of inorganic material with carbonate apatite identified in 99% of stones, phosphate in 88%, calcium in 87%, magnesium in 68%, struvite in 44%, oxalate in 38% and carbonate in 35%.

Hard salivary stones contain struvite more often, than stones of soft consistency. Large stones (>100mg) contained frequently more carbonate than small stones (<100mg). Stones from older patients (≥38 years) showed an almost significant trend toward more frequent presence of phosphate. The biochemical, inorganic composition of submandibular and parotid salivary stones is related to stone-related factors (size and consistency), probably to age, but not to patients gender.

Salivary stones consist, in addition to inorganic material, also organic material. Proteins contribute about 5% of the dry weight of submandibular salivary stones and they are mostly found in the core of the stone and sometimes on the surface. Salivary proteins such as lactoferrin, lysozyme and s-IgA have the property of clumping together and reinforcing each other in their specific actions. Possibly, this clumping of proteins could play a role in the formation of salivary stones. Therefore, in **Chapter 6**, twenty submandibular salivary

stones were examined for the presence of different salivary proteins. Using a combination of SDS-PAGE gel electrophoresis and Western blotting, α-amylase was found to be present in all stones, lysozyme in 95%, lactoferrin in 85%, s-IgA in 75%, MUC7 in 60%, complement C4 in 60% and C-reactive protein in 35%. The presence of, and co-occurrence of, lactoferrin, lysozyme, s-IgA and α-amylase in salivary stones was demonstrated by Enzyme-Linked Immuno Sorbent Assay (ELISA). Lactoferrin concentration in stones showed a significant, positive correlation with total protein concentration while lysozyme and s-IgA concentration did not correlate with total protein concentration.

Future research on the location of different proteins in salivary stones and the interactions between them is needed. This information may contribute to a better understanding of the pathogenesis of salivary stones.



NEDERLANDSE SAMENVATTING

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NEDERLANDSE SAMENVATTING

Speekselstenen of sialolieten zijn verharde, steenachtige verkalkingen die vooral ontwikkelen in de afvoergangen van een speekselklier en minder vaak in de klier zelf. Ze komen meestal voor in de afvoergang van de glandula submandibularis (84%) en minder vaak in die van de glandula parotidea (13%). In beide speekselklieren bevinden de speekselstenen zich veelal in de hoofdafvoergang en minder vaak in de kleine aftakkingen in de klier. Speekselstenen komen slechts sporadisch voor in het ductale systeem van de glandula sublingualis en de accessorische speekselkliertjes.

De aanwezigheid van een speekselsteen kan leiden tot een gedeeltelijke of volledige obstructie van de afvoergang. Hierdoor kunnen symptomen als recidiverende, maaltijd gerelateerde zwelling en pijn van de aangedane speekselklier optreden. Na verschillende episodes van deze maaltijd gerelateerde verschijnselen kunnen de zwelling en pijn min of meer continue aanwezig zijn. De ernst van de klachten kan variëren en wordt bepaald door de lokalisatie en grootte van de speekselsteen. Een speekselsteen hoeft geen klachten te veroorzaken en wordt soms als toevalsbevinding op een röntgenfoto van de kaak geconstateerd.

De ontstaanswijze van speekselstenen is onduidelijk. In de loop der jaren zijn verschillende hypothesen opgesteld die de etiologie en pathofysiologie van speekselsteenvorming probeerden te verklaren, zoals het samenklonteren van micro slijmpropjes tot een grote steenachtige verkalking en verschillen in de anatomie en de samenstelling van speeksel. Het vaker voorkomen van speekselstenen in de glandula submandibularis wordt mogelijk verklaart door de lengte en het kronkelige en opwaartse verloop van de hoofdafvoergang (ductus Whartoni) en de hogere viscositeit van speeksel van de glandula submandibularis vergeleken met speeksel van de glandula parotidea.

Het onderzoek dat wordt beschreven in dit proefschrift was hoofdzakelijk gericht op submandibulaire speekselstenen met speciale aandacht voor mogelijke factoren voor het ontstaan van een speekselsteen, het radiologische en klinische verschil in grootte van speekselstenen en de biochemische samenstelling van speekselstenen.

In **hoofdstuk 2** wordt op basis van de huidige wetenschappelijke literatuur een overzicht gegeven van de kennis over de etiologie, symptomen, biochemische samenstelling en de behandeling van speekselstenen. Er werd onder meer gevonden

dat wanneer de speekselproductie stijgt, bijvoorbeeld bij het denken of ruiken aan eten waardoor de intraglandulaire druk in de speekselklier stijgt, de pijn en zwelling van de aangedane klier toenemen. Speekselstenen bestaan uit een amorfe gemineraliseerde kern, omgeven door concentrische gelamineerde lagen van organische (collageen, proteïnen, aminozuren en koolhydraten) en anorganische stoffen (hydroxyapatiet, carbonaatapatiet, whitlockiet en brushiet).

Succesvolle verwijdering van speekselstenen is afhankelijk van informatie vooraf over de exacte locatie, grootte en de vorm van de stenen. In **hoofdstuk 3** is de nauwkeurigheid van een preoperatieve Cone Beam Computed Tomography (CBCT)-scan bij het bepalen van het volume van een speekselsteen onderzocht. Uit dit onderzoek bleek dat wanneer CBCT-scans gebruikt worden als diagnostisch hulpmiddel bij verdenking op submandibulaire speekselstenen de steen in werkelijkheid een fractie kleiner is dan bij het radiologisch onderzoek is bepaald. Dit is van belang omdat afkapwaarden voor de grootte van stenen worden gebruikt bij de keuze van het type behandeling bij steenverwijdering.

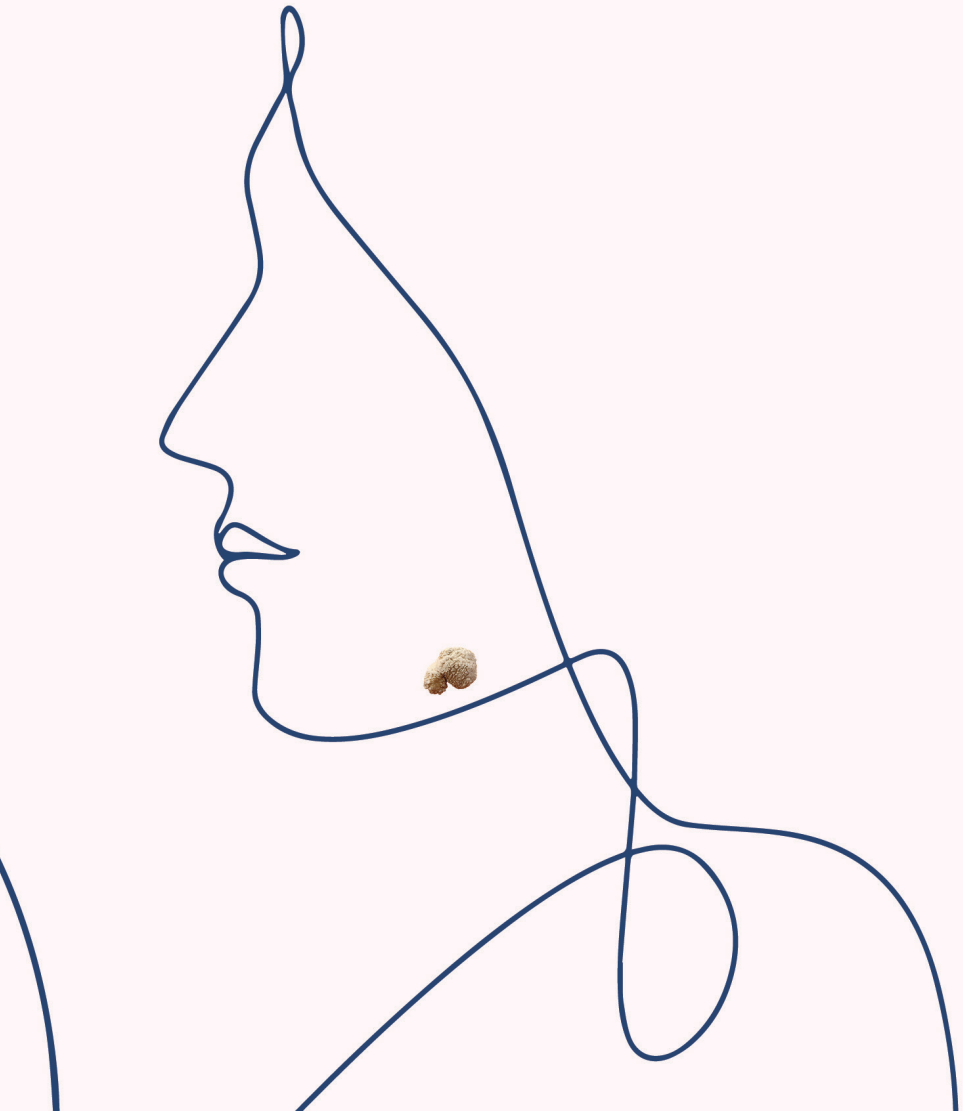
Over het verband tussen leefstijl factoren en het ontstaan van speekselstenen is weinig bekend. In **hoofdstuk 4** is de mogelijke relatie tussen het voorkomen van speekselstenen en systemische ziekten, gebruik van medicatie, roken en alcoholgebruik onderzocht. Hierbij bleek dat patiënten met speekselstenen significant frequenter antibiotica hadden gebruikt dan de controlegroep. Er werd geen verband gevonden tussen het optreden van speekselstenen en enkele vaak voorkomende systemische ziekten zoals hypertensie, diabetes mellitus en reumatoïde artritis. Roken en alcoholgebruik speelden geen of slechts een beperkte rol bij het ontstaan van speekselstenen.

In **hoofdstuk 5** werd de biochemische, anorganische samenstelling van submandibulaire en parotis speekselstenen bepaald en de mogelijke relatie tussen steen specifieke kenmerken en patiënt gerelateerde karakteristieken onderzocht. Speekselstenen blijken hoofdzakelijk samengesteld uit anorganisch materiaal waarbij carbonaatapatiet in 99% van de stenen werd geïdentificeerd, fosfaat in 88%, calcium in 87%, magnesium in 68%, struviet in 44%, oxalaat in 38% en carbonaat in 35%. Harde speekselstenen bevatten vaker struviet, dan stenen met een zachte consistentie. Grote stenen (>100mg) bevatten vaker carbonaat dan kleine stenen (< 100mg). Stenen van oudere patiënten (≥38 jaar) vertoonden een bijna significante trend naar frequentere aanwezigheid van fosfaat. De biochemische, anorganische samenstelling van submandibulaire en parotis

speekselstenen is gerelateerd aan steen gerelateerde factoren (grootte en consistentie), waarschijnlijk aan leeftijd, maar niet aan het geslacht van de patiënt.

Speekselstenen bestaan, naast anorganisch materiaal, ook voor een deel uit organisch materiaal. Eiwitten dragen voor ongeveer 5% bij aan het drooggewicht van submandibulaire speekselstenen en zij worden veelal gevonden in de kern van de steen en soms aan de oppervlakte. Speekseleiwitten zoals lactoferrine, lysozym en s-IgA hebben de eigenschap om samen te klonteren en elkaar te versterken in hun specifieke werking. Mogelijk zou deze samenklontering van eiwitten een rol kunnen spelen bij het ontstaan van speekselstenen. In **hoofdstuk 6** zijn daarom twintig submandibulaire speekselstenen onderzocht op de aanwezigheid van verschillende speekseleiwitten. Met behulp van een combinatie van SDS-PAGE gelelektroforese en Western blotting bleek in alle stenen α-amylase aanwezig, lysozym in 95%, lactoferrine in 85%, s-IgA in 75%, MUC7 in 60%, complement C4 in 60% en C-reactief proteïne in 35%. De aanwezigheid van, en het gelijktijdig voorkomen, van lactoferrine, lysozym, s-IgA en α-amylase in speekselstenen werd aangetoond door middel van Enzyme-Linked Immuno Sorbent Assay (ELISA). De lactoferrine concentratie in stenen toonde een significante, positieve correlatie met de totale eiwitconcentratie terwijl de concentratie lysozym en sIgA niet correleerde met de totale eiwitconcentratie.

Toekomstig onderzoek naar de locatie van de verschillende eiwitten in speekselstenen en de interacties tussen deze eiwitten is nodig. Deze informatie kan bijdragen tot een beter begrip van de pathogenese van speekselstenen.



AUTHOR CONTRIBUTIONS

A

OVERVIEW OF ARTICLES AND AUTHOR CONTRIBUTIONS

Chapter 2 - Salivary stones: symptoms, aetiology, biochemical composition and treatment.

Kraaij S, Karagozoglu KH, Forouzanfar T, Veerman ECI, Brand HS

Br Dent J. 2014 Dec 5;217(11):E23. doi: 10.1038/sj.bdj.2014.1054. PMID: 25476659.

Author contributions

Study design: SK, KHK, HSB

Data analyses and interpretation: SK, HSB

Manuscript draft: SK, KHK, TF, ECIV, HSB

Chapter 3 - Relationship between volume of submandibular salivary stones in vivo determined with Cone-Beam Computer Tomography and in vitro with micro-Computer Tomography.

Kraaij S, Brand HS, van der Meij EH, de Visscher JGAM

Med Oral Patol Oral Cir Bucal. 2021 Sep 1;26(5):e598-e601. doi: 10.4317/medoral.24605. PMID: 34415002; PMCID: PMC8412442.

Author contributions

Study design: SK, HSB, JGAMV

Data sampling: EHM, JGAMV

Data analyses and interpretation: SK, HSB, JGAMV

Manuscript draft: SK, HSB, EHM, JGAMV

Chapter 4 - Systemic diseases and the risk of developing salivary stones: a case control study.

Kraaij S, Karagozoglu KH, Kenter YAG, Pijpe J, Gilijamse M, Brand HS

Oral Surg Oral Med Oral Pathol Oral Radiol. 2015 May;119(5):539-43. doi:10.1016/j.oooo.2015.01.010. Epub 2015 Feb 3. PMID: 25753448.

Author contributions

Study design: SK, YAGK, HSB

Data sampling: KHK, YAGK, JP, MG

Data analyses and interpretation: SK, YAGK, HSB

Manuscript draft: SK, YAGK, HSB

Chapter 5 - Biochemical composition of salivary stones in relation to stone- and patient-related factors.

Kraaij S, Brand HS, van der Meij EH, de Visscher JGAM

Med Oral Patol Oral Cir Bucal. 2018 Sep 1;23(5):e540-e544. doi: 10.4317/medoral.22533. PMID: 30148468; PMCID: PMC6167104.

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Study design: SK, HSB, JGAMV

Data sampling: EHM, JGAMV

Data analyses and interpretation: SK, HSB, JGAMV

Manuscript draft: SK, HSB, EHM, JGAMV

Chapter 6 – Lactoferrin and the development of salivary stones: a pilot study.

Kraaij S, de Visscher JGAM, Apperloo RC, Nazmi K, Bikker FJ, Brand HS
Biometals. 2022 Nov 17. doi: 10.1007/s10534-022-00465-7. Epub ahead of print. PMID: 36396778.

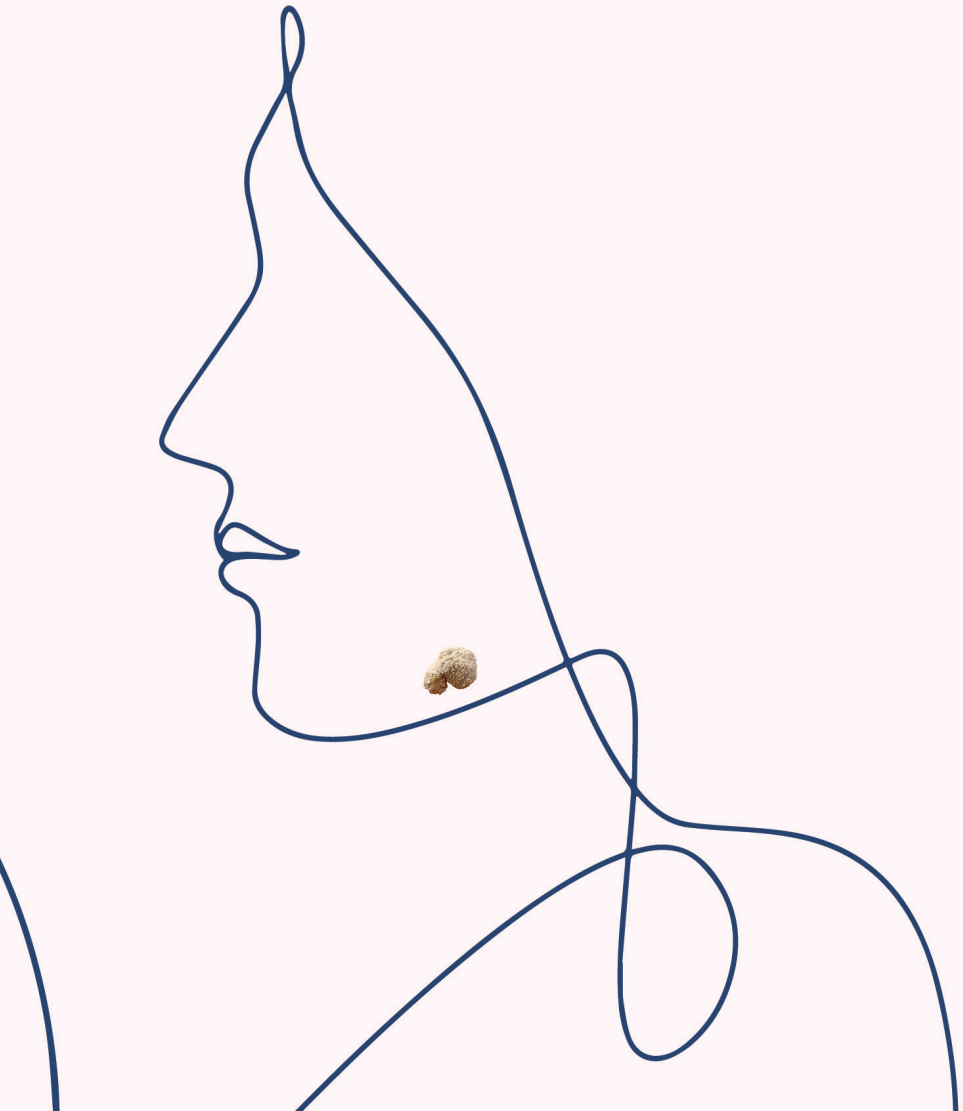
Author contributions

Study design: SK, HSB, KN

Data sampling: RCA, JGAMV

Data analyses and interpretation: SK, KN, HSB, FJB

Manuscript draft: SK, HSB, FJB, JGAMV



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AC

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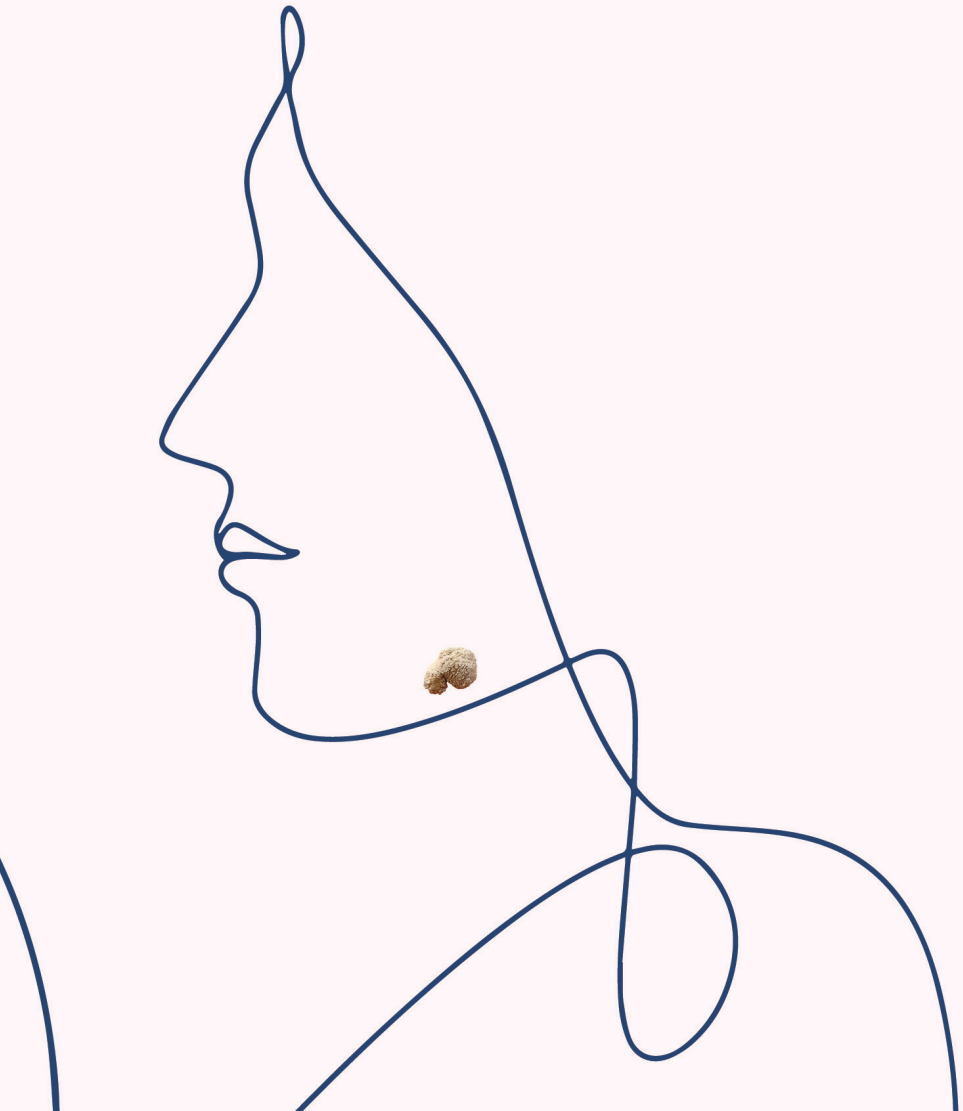

ABOUT THE AUTHOR

AB

OVER DE AUTEUR

Saskia Kraaij werd geboren op 29 maart 1987 te Amsterdam. In 2005 behaalde zij haar VWO-diploma, profiel Natuur & Gezondheid, aan scholengemeenschap Broklede te Breukelen. Haar studententijd startte in Nijmegen, waar zij haar propedeuse diploma Mondzorgkunde behaalde. In 2006 verhuisde zij (terug) naar Amsterdam om te beginnen aan haar opleiding Tandheelkunde aan het ACTA. In 2010 behaalde zij haar bachelor diploma welke in 2013 werd gevold door het masterdiploma. De wetenschap trok, zeker nadat zij tijdens haar master thesis een onderzoek had opgezet naar speekselstenen. Besloten werd om na het afstuderen, als gastmedewerker aan het ACTA, het afstudeeronderzoek uit te bouwen naar een promotietraject.

Naast het promotietraject werkt(e) Saskia fulltime in verschillende tandartspraktijken in de regio Amsterdam als tandarts.