Diagnosis of cardiovascular disease by detection of hs-cTnT and NT-proBNP in saliva – a feasibility study

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Summary

Aim
To pilot test if saliva can be used for analysis of high-sensitivity cardiac troponin T (hs-cTnT) and N-terminal pro-B-type natriuretic peptide (NT-proBNP). Also, as a secondary aim, to compare the levels of these biomarkers in saliva and plasma among patients suffering from acute myocardial infarction (AMI).

Methods
10 patients with STEMI or NSTEMI were sex and age matched to 10 controls without any self-reported heart-episode (i.e. ACS, uncompensated hypertension or heart failure) within the last 12 months. All participants provided stimulated saliva samples. In addition, blood samples were obtained from the patients. Samples were analyzed for the presence of hs-cTnT and NT-proBNP using commercially available plasma assays. Saliva samples were analyzed in duplicates in order to assess the imprecision of the methods used.

Results
hs-cTnT was not detectable (< 3.00 ng/L) in saliva in the majority of cases (n = 16). Controls had slightly higher salivary mean hs-cTnT than the patients (6.0 ng/L and 5.1 ng/L respectively, n = 4).

NT-proBNP concentrations were significantly higher in patient saliva when compared to controls (median 48.9 ng/L and 31.5 ng/L respectively, n = 20, p = 0.005). Among the patients there was a tendency towards a moderate correlation between plasma and saliva NT-proBNP concentrations, however this was not statistically significant (n = 10, r_s = 0.345, p = 0.328).

The imprecision of the hs-cTnT assay in saliva was 12.3 CV (%). With regard to NT-proBNP in saliva, the imprecision was 11.0 CV (%) and 5.3 CV (%) for controls and patients respectively.

Conclusions
In this pilot study a majority of the saliva samples had an hs-cTnT concentration below the limit of detection (< 3.00 ng/L). Salivary NT-proBNP was significantly higher among patients with AMI when compared to healthy controls. Further, in AMI patients, there is a tendency towards a moderate correlation between plasma and saliva NT-proBNP concentrations although not reaching statistical significance.
Sammanfattning (summary in Swedish)

Syfte
I form av en pilotstudie undersöka om saliv kan användas för att analysera högkänsligt hjärttroponin T (hs-cTnT) och N-terminal pro-B-type natriuretic peptide (NT-proBNP). Därutöver att studera markörernas koncentrationer i saliv i jämförelse med plasma bland patienter med hjärtinfarkt.

Metod

Resultat
hs-cTnT var inte detekterbart (< 3,00 ng/L) i majoriteten av salivproverna (n = 16). Kontrollproverna uppsvisade något högre medelkoncentration hs-cTnT jämfört med patientproverna (6,0 ng/L respektive 5,1 ng/L, n = 4).

NT-proBNP var signifikant högre i saliv från patientgruppen jämfört med kontrollgruppen (median 48,9 ng/L respektive 31,5 ng/L, n = 20, p = 0,005). En tendens till en moderat korrelation mellan plasma och saliv med avseende på NT-proBNP-koncentration påvisades, denna var emellertid ej statistiskt signifikant (n = 10, r_s = 0,345, p = 0,328).

Variationen mellan dubbelproverna (CV) var för salivanalys av hs-cTnT 12,3 %. Gällande NT-proBNP var motsvarande variation 11,0 % och 5,3 % för kontrollgruppen respektive patientgruppen.

Slutsats
En majoritet av salivproverna i denna pilotstudie hade en hs-cTnT-koncentration under analysmetodens detektionsgräns (< 3,00 ng/L). NT-proBNP-koncentrationen var signifikant högre i saliv hos patienter med hjärtinfarkt jämfört med friska kontroller. Utöver detta sågs en icke statistiskt signifikant tendens till korrelation mellan plasma och saliv med avseende på NT-proBNP bland hjärtinfarktpatienter.
Authors’ contributions
Both authors contributed to an equal extent thorough all stages in the preparation of this report.

To specify in detail, Jakob Malmberg participated in designing the study, in the process of obtaining ethical approval, in establishing contacts crucial for the execution of the project, in patient and control sample collection and in handling of samples prior to laboratory analysis. Additionally, Jakob preformed the statistical analysis and, finally, contributed to all sections of this report.

Turid Tidblom participated in designing the study, in the process of obtaining ethical approval, in establishing contacts crucial for the execution of the project, in patient and control sample collection and in handling of samples prior to laboratory analysis. Additionally, Turid preformed the statistical analysis and, finally, contributed to all sections of this report.
Introduction

Modern medicine relies, to a large extent, on the observation of disease states based on recording of various parameters related to the homeostasis of the body. The physical examination of the patient reveals some of the basic parameters, for example heart rate, blood pressure and body temperature. Data attained through the physical examination are some of the earliest examples of biomarker use. A biomarker is defined by the National Institutes of Health (NIH) as:

“a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.

This definition is thus broad and includes, in addition to physical parameters, laboratory tests, radiographic evaluation and other modalities used to monitor the body. The development of the concept using bodily fluids to determine presence or absence of disease is credited to the ancient Greeks. Most of the tests conducted at the time used the urine of the patient. The nineteenth and twentieth century saw significant improvements in the ability to collect and analyze biologic material. A systematic approach in establishing reference levels for different biomolecules followed. Blood (and to some extent urine) evolved to the bio fluid of choice for biomarker discovery and clinical use. Healthcare today is dependent on laboratory tests for detection of disease, assessment of response to treatment and determination of prognosis. Advancements in laboratory procedures are constantly improving the sensitivity and specificity of the tests while also simplifying sample collection and handling (1).

During the last decades there has been an increased interest in other bio fluids that offer a non-invasive, safe and simple sample collection. These characteristics of a bio fluid are of special importance when considering screening programs of a large population. Saliva has shown great promise in this regard. Whole saliva (WS) includes both the exocrine secretions of the salivary glands and the gingival crevicular fluid (GCF) implicating that many biomarkers found in blood also can be detected in saliva (2).

Saliva as a diagnostic tool

Whole saliva (WS) is the fluid that lines the mucosa of the oral cavity. It aids in mastication, speech and is crucial to the integrity of the dental hard tissues. The main components of saliva are water, peptides, proteins, hormones, sugars, lipids and various electrolytes. The exact composition of WS at any given time is dependent on the exocrine secretions from the salivary glands and the non-exocrine (or non-salivary) components. There
are three major salivary glands: the parotid, the submandibular and the sublingual glands. In addition, a large number of minor salivary glands are scattered in the oral cavity. The non-exocrine components of saliva are mainly derived from the gingival crevicular fluid (GCF) and the nasal and bronchial secretions. Constant turnover of the epithelium of the oral mucosa, lesions in the mucosa and microbiologic activity in the mouth also contribute to the non-exocrine component of WS (3).

The findings above support the notion that saliva may indeed be "the mirror of the body" and may be used as an alternate bio fluid for various diagnostic applications. Concomitantly with the development of highly refined methods for the qualitative and quantitative analysis of small sample volumes considerable efforts have been made to evaluate the true diagnostic potential of saliva. This expands the application of “saliva diagnostics” beyond the diseases of the oral cavity (i.e. periodontitis and dental caries) to include the entire physiologic system (4). Presently, this has materialized into several commercially available products such a rapid test for HIV (OraQuick®), tests for steroid hormones and tests for alcohol and drug abuse (5).

In line with the effort to evaluate the diagnostic potential of saliva, Loo and colleagues compared the proteome of saliva and plasma and found a 27 % overlap. The overlap increased to 40 % when specifically targeting proteins suggested as markers for diseases such as cancer, cardiovascular disease and stroke (3).

The concentration of a particular biomarker found in blood may be expected to be substantially lower in saliva, thus requiring refined laboratory techniques to detect even minute levels of the biomarker of interest. This disadvantage is, however, balanced by the non-invasive nature of saliva collection and the ease of sample collection (not requiring particular training or special facilities) (5). This is especially important when considering screening of large populations, for example to aid in risk assessment. Nevertheless, sample collection, handling and storage may be complicated by the inherent photolytic activity of WS (6). Underlining the importance of further assessing the accuracy of saliva based diagnostics compared to current golden standards.

**Plasma and saliva biomarkers for acute coronary syndrome**

In 2008, 17.3 million people died due to cardiovascular diseases (CVD) making CVD the number one cause of death worldwide (7). The high prevalence of CVD is not only a major source of individual suffering but
also exerts a substantial burden on the healthcare system. In the European Union alone, healthcare expenditure and productivity loss due to CVD is estimated to €169 billion yearly (8). The entity of CVD encompasses all disorders of the heart and blood vessels as schematically illustrated in figure I.1.

**FIGURE I.1 Pathogenesis of CVD**

Acute coronary syndrome (ACS) is one of the most severe and life threatening CVDs. This is when a prolonged ischemic injury leads to necrosis of cells in the myocardium, defined as myocardial infarction.

In 2007, the Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction published a universal definition of acute myocardial infarction (AMI). Their definition emphasized the use of serological biomarkers in addition to the conventional clinical parameters. For review, see Thygesen et al. 2007 (10).
PLASMA BIOMARKERS FOR DETECTION AND MONITORING OF ACUTE CORONARY SYNDROME

Due to the life threatening nature of ACS, significant efforts have been directed towards developing a reliable and rapid method to verify true events of myocardial infarction. Clinical management of the patient’s condition is predominantly based on clinical, ECG and laboratory findings (10). These parameters are included in various clinical decision making matrixes to guide treatment and follow up. Also, additional parameters, such as plasma-based biomarkers, may be used to assess short- and long-term prognosis for example in patients suffering from decompensated heart failure as a consequence of ACS (11). Moreover, plasma-based biomarkers have been shown to improve risk stratification among elderly with no previous cardiovascular event (12).

Several different plasma-based biomarkers have been suggested as indicative of the existence of and severity of a cardiovascular event. The relationship between various plasma-based biomarkers and the cardiovascular disease process is illustrated in figure I.2.

FIGURE I.2 Pathogenesis of CVD in relation to expression of relevant biomarkers

Adopted from Kehl et al. (2011) (9)
Cardiac troponin (cTn) is regarded as the current golden standard for the detection of myocardial injury. The cTn protein is involved in contraction and relaxation of striated muscle and is composed of three subunits: C, (cTnC), I (cTnI) and T (cTnT). cTnC is expressed in both cardiac and skeletal muscle while cTnT and cTnI are expressed only in cardiac muscle cells. Hence, assays used in the setting of ACS are specific to cTnT and/or cTnI (13). Both are limited in diagnostic capability at presentation, however, this capability has been shown to improve rapidly the first 12 hours (14). Nevertheless, this limitation has sparked interest in developing high-sensitivity troponin assays. The performance of these assays has been shown to improve the diagnostic accuracy at presentation significantly with an excellent negative predictive value (NPV) in the range of 97-99%, thereby reducing the initial diagnostic uncertainty associated with conventional troponin assays (15).

In an effort to expand capability of biomarkers in ACS beyond diagnostics to predictions, several multi biomarker assays have been suggested. Some have centered on the use of natriuretic peptides, hormones produced in response to injury of the heart. The N-terminal pro-B-type natriuretic peptide (NT-proBNP) has been proposed as a diagnostic biomarker for ACS. However, the accuracy is inferior to conventional troponin cTnT but when the two are combined this yields a higher accuracy than cTnT alone (9). The major advantage of including NT-proBNP in the assay is the prognostic value of this biomarker. A positive correlation between high NT-proBNP and cTnT levels at presentation and poor prognosis has been established (16). Furthermore, using assays including cystatin C and C-reactive protein (CRP) in addition to NT-proBNP and cTnI, provided improved risk stratification among elderly without any previous cardiovascular event, suggesting the use of cardiac biomarkers for the risk assessment of patients with no previous history of cardiac disease (12).

In summary, cardiac troponin (subunits I and T) is the golden standard among serological tests for verification of suspected myocardial injury. Limitations in the diagnostic potential of conventional cardiac troponin at presentation have been partly overcome with the introduction of high-sensitivity cardiac troponin tests. NT-proBNP provides information about the short- and long-term prognosis of a cardiac event. Further, there are indications that inclusion of additional plasma biomarkers such as cystatin C and C-reactive protein (CRP) may improve risk stratification among individuals with no history of cardiac disease.
SALIVARY BIOMARKERS FOR DETECTION AND MONITORING OF ACUTE CORONARY SYNDROME

As discussed above, saliva is a diagnostic fluid of great potential. Many biomarkers found in plasma may also be detected in saliva albeit at much lower concentrations (3). Due to this, saliva based diagnostics require highly sensitive laboratory approaches. When these are available the advantages of using saliva as a diagnostic medium are apparent, namely ease of collection and the non-invasiveness of the test. However, the robustness of the procedure and the influence of various parameters such as sampling protocol, oral diseases and storage have not yet been thoroughly evaluated.

In the context of ACS, Floriano and colleagues conducted an assay of 21 proteins in saliva collected from 41 AMI patients and 43 controls. While this study was aimed at evaluating the use of a saliva-based nano-biochip for AMI, it also provided one of the first insights into the feasibility of saliva-based detection of ACS. Interestingly, using saliva cTnI in conjunction with saliva creatine-kinase MB isoenzyme (CK-MB), brain-natriuretic peptide (BNP) and myoglobin (MYO) yielded diagnostic accuracy far below that of plasma based assays. However, the authors speculated that this might be due to the relatively poor sensitivity of the laboratory test used (17).

In 2011, Mirzaii-Dizgah et al. studied the correlation between plasma and saliva CK-MB. This study showed a strong correlation between unstimulated saliva and plasma values of CK-MB and the correlation was true among AMI patients as well as among healthy controls (18). In a very recent follow-up on the saliva CK-MB study, the correlation between saliva and plasma high-sensitivity cardiac troponin T (hs-cTnT) was studied using an unspecified ELISA assay. A significant, moderate, correlation between plasma and saliva hs-cTnT was demonstrated, true for both AMI patients and healthy controls, regardless of whether the saliva was stimulated or unstimulated. The authors concluded that saliva hs-cTnT might be used as an alternative to plasma hs-cTnT (19). However, the reliability, under clinical conditions, of the saliva hs-cTnT method used can be questioned due to the strict study regime which required participants to refrain from eating, drinking, smoking and brushing of teeth 1 h prior to sample collection. Also, no data on the precision of the ELISA assay used is available.

With regard to the presence of NT-proBNP in saliva it has been shown to be quantifiable in human unstimulated saliva in patients suffering from heart failure (HF). In a study by Foo et al. a custom-made immunoassay was used for the analysis of the saliva samples. This assay was evaluated against a commercially available assay with excellent results.
Further, saliva NT-proBNP concentrations among the HF patients were higher when compared to healthy controls. Significantly, no correlation between saliva and plasma NT-proBNP concentrations could be demonstrated (20).

In the light of the few available studies in the field of saliva diagnostics for CVD, the present study is designed to evaluate the feasibility of using commercially available plasma-based assays for detection of saliva hs-cTnT and NT-proBNP. Further, the precision of these methods will be assessed and any correlation between saliva and plasma hs-cTnT and NT-proBNP analyzed.

Aim
The primary aim of this study is to test the hypothesis that saliva can be used for analysis of high-sensitivity cardiac troponin T (hs-cTnT) and N-terminal pro-B-type natriuretic peptide (NT-proBNP). A secondary aim is to compare the levels of these biomarkers in saliva and plasma among patients suffering from AMI.
Materials and Methods

Participants
For the purpose of the study, 10 patients (“patient group”) admitted to the Department of Cardiology at Karolinska University Hospital (Huddinge, Sweden) were recruited.

The inclusion criteria for the patient group were a diagnosis of ST elevation myocardial infarction (STEMI) or non-ST elevation myocardial infarction (NSTEMI) and elevated hs-cTnT in plasma at the time of admittance. Patients deemed medically unstable and/or unable to comply with the study protocol were excluded from the study.

An additional 10 participants (“control group”) without any self-reported heart-episode, such as ACS, uncompensated hypertension or heart failure, within the last 12 months were recruited to act as controls. Controls were recruited among patients seeking dental care at the Department of Dental Medicine at Karolinska Institutet (Huddinge, Sweden). Controls were matched to the patient group with regard to sex and age (± 3 years).

Written informed consent was obtained from all patients and controls. The regional ethical review board in Stockholm, Sweden approved the study protocol (2012/238-31/2).

Sample collection
Written informed consent was obtained from all participants. Current medications were documented based on data in the medical record. Participants were then asked to report any tobacco habits. Among the participants in the patient group a diagnosis of STEMI or NSTEMI and elevated hs-cTnT was confirmed using medical record data.

The number of teeth (restored and intact) was recorded and the surrounding gingiva inspected for signs of inflammation. The degree of gingival inflammation was scored using the Modified Gingival Index (MGI) as described by Lobene and colleagues (21). The same examiner did all the assessments of the MGI.

Participants were then asked to chew on a natural paraffin pellet (Orion Diagnostica, Finland) and then swallow all oral fluid before sample collection started, during which, the participant was asked to expectorate all oral fluid into a plastic cup. The stimulated whole saliva was then transferred to a graded plastic tube (Sarstedt, Germany). The sample collection was stopped when approximately 3 mL of stimulated whole saliva had been obtained by the method described. In the patient group, the
attending nurse drew 3-4 mL of venous blood following the saliva sample collection. Blood was drawn from the existing peripheral venous catheter of the patient using a standard EDTA coated tube (Vacuette®, Greiner bio-one, Switzerland).

All samples from the patient group were collected within 48 hrs of admittance to the hospital.

All saliva and plasma samples were centrifuged within 1 h of collection at 3800 g at 4 °C for 10 minutes. Subsequently, the supernatants of saliva and plasma were divided into aliquots and stored at -70 °C for later analysis.

**Laboratory analysis**

An independent laboratory analyzed the plasma and saliva samples (Aleris Medilab, Täby, Sweden).

Prior to the analysis of plasma and saliva the aliquots were thawed in room temperature and centrifuged before transferring the supernatant to test tubes as recommended by the manufacturer of the assays used (see below). To standardize the procedure, all samples were analyzed approximately 30 minutes after removal from cold storage.

For the analysis of plasma and saliva hs-cTnT the Troponin T hs STAT assay in a Cobas e 411 analyzer (Roche Diagnostics, Germany) was used.

For the analysis of plasma and saliva NT-proBNP the NT-proBNP assay in an Immulite 2000 analyzer (Siemens Medical Solutions, Germany) was used.

All saliva samples were analyzed in duplicates to assess the precision of the methods used. In cases where one duplicate was below the limit of blank (LoB) and the other was above LoB a third analysis was performed. The arithmetic mean of the respective analyses was then used for any further calculations.

**Statistical analysis**

All statistical calculations were performed using SPSS version 21 (IBM, USA). Intra- and intergroup characteristics are given as mean ± SD. Non-normally distributed data is presented as median and interquartile range (IQR).

The precision of the saliva based assays for hs-cTnT and NT-proBNP was determined by calculating the coefficient of variation (CV). Intergroup differences were tested using Mann-Whitney U test and finally any
correlation between plasma and saliva biomarker concentrations was assessed using Spearman’s Rank-Order Correlation test. P-values below 0.05 were considered statistically significant.
Results

Table R.1 summarizes patient and control group characteristics at the time of sample collection. There were no major differences with regard to age, number of remaining teeth, gingival inflammation and current tobacco use. However, the patient group had a greater mean number of medications when compared to the control group.

**TABLE R.1 Group characteristics at sample collection (*mean ± SD)**

<table>
<thead>
<tr>
<th></th>
<th>Patient group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>7:3</td>
<td>7:3</td>
</tr>
<tr>
<td>Age</td>
<td>68.7 ± 11.4*</td>
<td>68.9 ± 11.1*</td>
</tr>
<tr>
<td></td>
<td>(54-88)</td>
<td>(53-85)</td>
</tr>
<tr>
<td>Number of teeth</td>
<td>21.9 ± 9.1*</td>
<td>21.9 ± 6.1*</td>
</tr>
<tr>
<td>Modified gingival index (MGI)</td>
<td>1.0 ± 0.8*</td>
<td>1.4 ± 0.8*</td>
</tr>
<tr>
<td>Number of medications</td>
<td>12.2 ± 3.7*</td>
<td>1.4 ± 1.4*</td>
</tr>
<tr>
<td>Current tobacco use</td>
<td>10 %</td>
<td>20 %</td>
</tr>
</tbody>
</table>

**Plasma and saliva hs-cTnT**

In the patient group the mean plasma hs-cTnT was 613.7 ng/L (SD ± 588.5). Saliva hs-cTnT concentration above 3.00 ng/L, the “limit of blank” (LoB), was only detected in 4 samples, in 2 samples from the patient group and 2 samples from the control group. Among the control samples, 3 duplicates were inconclusive with regard to the relation to the LoB. In these cases a third assay was performed. The mean saliva hs-cTnT concentration was higher among the controls, 6.0 ng/L (SD ± 3.1), compared to 5.1 ng/L (SD ± 1.7) in the patient group. An overview of the findings is available in table R.2 below.
TABLE R.2 Plasma and saliva hs-cTnT (mean ± SD)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma (ng/L)</strong></td>
<td></td>
</tr>
<tr>
<td>613.7 ± 588.5 (n = 10)</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Saliva (ng/L)</strong></td>
<td></td>
</tr>
<tr>
<td>5.1 ± 1.7 (n = 2)*</td>
<td>6.0 ± 3.1 (n = 2)*</td>
</tr>
</tbody>
</table>

*Of the saliva samples available, 16 had hs-cTnT below the LoB (limit of blank) of the assay. This corresponds to a concentration of < 3.00 ng/L.
ND = No data.

Imprecision for the saliva based hs-cTnT assay was 12.3 CV (%) based on duplicates of the 4 samples with hs-cTnT concentrations above LoB. Due to the small number of available samples further statistical analysis was not performed.

**Plasma and saliva NT-proBNP**
Among the patients, the median plasma NT-proBNP concentration was 1176.5 ng/L. In contrast to hs-cTnT, NT-proBNP was readily detectable in saliva from all patients and controls. The median salivary NT-proBNP was 48.9 ng/L and 31.5 ng/L for patients and controls respectively (p = 0.005). The observations with regard to NT-proBNP are summarized in table R.3 and illustrated in figure R.4.
### TABLE R.3 Plasma and saliva NT-proBNP presented as median (IQR)

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Control group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (ng/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1176.5 (500.0-3020.8)</td>
<td>ND</td>
<td>0.005*</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva (ng/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48.9 (44.8-68.4)</td>
<td>31.5 (24.6-41.6)</td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>(n = 10)</td>
<td></td>
</tr>
</tbody>
</table>

* Indicates statistically significant differences using Mann-Whitney U test.
ND = No data.

### FIGURE R.4 Patient and control saliva NT-proBNP concentration boxplot

Imprecision for the saliva based NT-proBNP assay was 11.0 CV (%) for the controls (n = 10) and 5.3 CV (%) for the patients (n = 10).

To evaluate any correlation between plasma and salivary NT-proBNP concentrations among patients, a scatterplot of the data was created (figure R.5).
Further, a Spearman’s rank order correlation was performed. There was a tendency towards a moderate, positive, correlation between plasma NT-proBNP and salivary NT-proBNP \((n = 10, r_s = 0.345, p = 0.328)\) although this was non-significant.
Discussion

The present study was designed to determine the feasibility of accurately detecting and quantifying hs-cTnT and NT-proBNP in human stimulated saliva. Samples were collected from 10 patients suffering from AMI and from an additional 10 healthy controls. Despite the small study population in this pilot study, several important trends are present within the available data.

This study utilized two commercially available plasma-based assays for the detection of salivary hs-cTnT and NT-proBNP. The hs-cTnT assay was not successful in detecting salivary hs-cTnT in the majority of cases. However, in the case of NT-proBNP, all salivary samples had a measurable amount of the biomarker in question. This was true for both patients and controls.

It is not clear why the hs-cTnT assay failed in detecting salivary hs-cTnT. It can be speculated that the assay, which is intended for plasma mediums, is unable to handle saliva specimens correctly. To investigate this theory, saliva from one of the controls with salivary hs-cTnT below LoB was spiked with a known concentration of hs-cTnT and then repeatedly analyzed over 24 hours. This produced reliable data concerning the hs-cTnT concentration in saliva. The salivary hs-cTnT dropped slowly over the 24 hours confirming the inherent proteolytic activity of saliva, but not to an extent explaining the very low hs-cTnT concentrations in our samples (6). With these indications in mind, other possible reasons for the failure in detecting hs-cTnT in saliva are: (a) there is no exchange of hs-cTnT between plasma and saliva (b) saliva hs-cTnT binds to surfaces of the oral cavity and/or microorganisms that colonize it, thereby reducing the amount of free hs-cTnT in saliva (c) the epitope of the hs-cTnT molecule is modified during sample handling and/or subsequent cold storage in a saliva medium and (d) combinations of the above. Since total salivary protein was not assessed in the present study, the influence of the saliva flow rate (i.e. total protein concentration of the sample) has not been evaluated.

Regardless of the reasons for failure, our results are in conflict with the findings of Mirzaii-Dizgah et al. (19). In their study, hs-cTnT was quantifiable in stimulated saliva from patients and controls at approximately a 3:1 (plasma:saliva) ratio. Moreover, hs-cTnT concentrations in stimulated saliva were significantly higher among patients when compared to healthy controls. Within the group of healthy controls, hs-cTnT concentrations were higher in stimulated saliva compared to unstimulated saliva. The opposite relationship was reported for the group of patients.

There are several important differences between our study and the study of Mirzaii-Dizgah et al. First and foremost, the study population in the Mirzaii-
Dizgah et al study was three times larger than in our study, encompassing a total of 60 participants. All participants in their study had to follow a standardized sample collection protocol similar to the protocol detailed by Henson & Wong (22), while in our study samples were collected under less ideal (i.e. clinical) conditions.

In terms of laboratory analysis, Mirzaii-Dizgah et al. used an ELISA based method. No data is available regarding the specificity and precision of this assay. It is nevertheless interesting to note that Mirzaii-Dizgah et al. reported an average plasma hs-cTnT of 25.7 ng/L (SD ± 30.7) for the group of healthy controls. This value must be considered unusually high as the 99th percentile for healthy individuals of the Troponin T hs STAT assay is 14 ng/L in plasma (23). Since plasma samples were not collected from the controls in the present study, no data is available for a complete comparison of the two control study populations. When comparing the two patient study populations, it is noteworthy that, although the patient group in our study had a substantially higher mean hs-cTnT in plasma, the assay was unable to detect any salivary hs-cTnT in most cases. This is in contrast to the findings of Mirzaii-Dizgah et al. Their patients had a mean plasma hs-cTnT not only considerably lower but also reported a mean concentration of hs-cTnT in stimulated saliva much higher than in our study (219.5 ng/L vs. 24.9 ng/L and 613.7 ng/L vs. 5.1 ng/L for plasma and saliva respectively).

In the assay of NT-proBNP, all samples had a quantifiable amount of this biomarker. Additionally, the salivary NT-proBNP was significantly higher among patients when compared to the controls. A trend towards a moderate correlation between plasma and salivary NT-proBNP was identified but did not reach statistical significance.

To the best of our knowledge, only one previous study regarding salivary NT-proBNP has been published (20). Foo and colleagues studied salivary NT-proBNP among 45 patients suffering from heart failure and 40 healthy controls using a non-commercially available AlphaLISA assay. To evaluate the performance of the assay, the group undertook a thorough evaluation of performance characteristics and also compared the AlphaLISA assay to a commercially available assay. The main findings of their study were a significantly higher salivary NT-proBNP in patients compared to controls, which is in accordance with our findings. Interestingly, all controls in the study by Foo et al. had a salivary NT-proBNP concentration below the “limit of detection” (LoD) at 16 ng/L. Based on these findings it was speculated that the presence of salivary NT-proBNP was specific to heart failure. Our study demonstrated a median salivary NT-proBNP of 31.5 ng/L among the controls which is in disagreement with this conclusion. The marked difference in salivary NT-proBNP among healthy controls of the
studies may be due to the substantially younger controls of Foo et al. and the older controls of our study (mean age 56 years vs. 69 years respectively). This speculation is supported by the fact that studies have shown that NT-proBNP concentrations in plasma increase with age (24). Other explanations for the higher salivary NT-proBNP concentrations in our assay may be that it picked up on an unidentified localized production of NT-proBNP. The concept of salivary NT-proBNP as a composite of plasma-transudate and local production therefore warrants further investigation. The specificity in saliva mediums of the assay used in the present study has not been evaluated further limiting comparisons between the two studies.

In terms of correlation between plasma and salivary NT-proBNP, Foo et al. found no correlation between plasma and salivary NT-proBNP in heart failure patients. Among the AMI patients in our study, a non-significant tendency towards a moderate correlation was demonstrated between plasma and salivary NT-proBNP. As outlined above, the implication of this is, however, limited especially due the small sample material in our study.

The present study set out to pilot test the feasibility to detect, quantify and correlate plasma and salivary hs-cTnT and NT-proBNP among AMI patients and healthy controls. While the study failed to detect salivary hs-cTnT, results were more promising with regard to NT-proBNP. It was shown that NT-proBNP not only can be detected in saliva but also is quantifiable and tends to correlate with plasma NT-proBNP concentrations. There is a small but increasing amount of evidence suggesting that saliva is a possible source of cardiac biomarkers. Our study tested one “diagnostic” biomarker (hs-cTnT) and one “predictive/prognostic” biomarker (NT-proBNP). As mentioned above, the latter was more successful. This may be of importance as it can be argued that the future of saliva diagnostics is not in diagnostics per se but instead in prediction of disease e.g. screening. There are several good reasons for a shift from “saliva diagnostics” to “saliva disease prediction / saliva risk assessment”.

CVD is the most common cause of death in the European Union (EU), estimated to kill nearly 2 million people per year within the region (25). The health expenditure and the productivity loss due to CVD in EU member states is estimated to €169 billion. Roughly, a quarter of this cost is attributed to coronary heart disease including ACS (8). Reducing deaths, suffering and the economic burden of CVD requires a combination of population-based prevention and identification of high-risk individuals as outlined in the European Heart Health Charter (25). Examples of population-based strategies are smoking cessation, exercise, and a balanced diet. The identification of high-risk individuals uses risk factors such as age,
sex, blood pressure and smoking. The role of plasma biomarkers in risk stratification is at present very small.

The limited usefulness of biomarkers in risk assessment is partly due to difficulties in identifying markers that add additional power to the existing models (26). Emerging evidence, however, suggest that this difficulty may be overcome by multimarker assays. An example of the latter is a cTnI/NT-proBNP/cystatin/CRP-assay that improved risk stratification over conventional methods among elderly men (12). This exact combination has, to the best of our knowledge, not been evaluated in saliva.

An additional problem with including biomarkers in risk stratification protocols is the additional cost that it incurs. Especially considering that a considerable part of the population in the developed countries can be considered being at “high risk”. In the perspective of cost-effectiveness saliva based methods may be the answer to the need of detecting and monitoring individuals at high risk for ACS. The ease of saliva collection makes it ideal both for the development of these assays and for the use in the clinical and also in the non-clinical setting. Saliva is thus not only a fluid of diagnostic capability but also one of predictive potential. These possible capabilities warrant further investigation regarding, for example, the presence of the biomarkers in saliva, the correlation between plasma and saliva biomarker concentrations and optimal saliva sample handling. Finally, large-scale studies are needed to evaluate the utility of various multi-marker saliva assays. In the long-term perspective saliva based diagnostics/prediction may well aid in tailoring treatment for the prevention of life threatening CVD-events. From patients to policy makers, prevention is always better than cure.

Conclusions
In this pilot study a majority of the saliva samples had an hs-cTnT concentration below the limit of detection (< 3.00 ng/L). Salivary NT-proBNP was significantly higher among patients with AMI when compared to healthy controls. Further, in AMI patients, there is a tendency towards a moderate correlation between plasma and saliva NT-proBNP concentrations although not reaching statistical significance.
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