

UNIVERSITY OF IOANNINA

SCHOOL OF HEALTH SCIENCES

FACULTY OF MEDICINE

2nd DEPARTMENT OF CARDIOLOGY



THESIS: « NOVEL BIOMARKERS IN HEART FAILURE PATIENTS»

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SUPERVISOR: LAMPROS K. MICHALIS, PROFESSOR OF CARDIOLOGY

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IOANNINA GREECE, JULY 2026

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ΠΕΡΙΛΗΨΗ

Σκοπός: Έχουν καθιερωθεί συγκεκριμένοι βιοδείκτες στο αίμα ως διαγνωστικοί και προγνωστικοί σε ασθενείς με καρδιακή ανεπάρκεια (ΚΑ). Οι πιο σημαντικοί και ευρέως χρησιμοποιούμενοι είναι τα νατριουρητικά πεπτίδια (NT-proBNP και BNP). Σε αυτή τη διατριβή, στόχος μας ήταν να διερευνήσουμε τον ρόλο των βιοδεικτών στο σίελο (πρωτίστως του NT-proBNP και δευτερευόντως της κορτιζόλης) στη διαχείριση της οξείας και χρόνιας ΚΑ (διάγνωση, διαστρωμάτωση κινδύνου και ανταπόκριση στη θεραπεία). Διερευνήθηκε επίσης η συσχέτιση των βιοδεικτών του σιέλου και του ορού.

Μέθοδοι: Συμμετείχαν ενενήντα επτά άτομα: 36 παχύσαρκοι ή υπερτασικοί με δύσπνοια αλλά χωρίς διάγνωση ΚΑ (χωρίς ηχοκαρδιογραφικά σημεία ΚΑ ή αυξημένα επίπεδα NT-proBNP ορού), οι οποίοι χρησίμευσαν ως ομάδα ελέγχου, 12 ασθενείς με χρόνια ΚΑ (CHF) και 49 ασθενείς με οξεία ΚΑ (AHF - de novo ή μη αντιρροπούμενη) που απαιτούσαν εισαγωγή στο νοσοκομείο. Καταγράφηκαν αρκετά κλινικά, απεικονιστικά και εργαστηριακά δεδομένα σε όλους τους συμμετέχοντες στα ακόλουθα χρονικά σημεία: κατά την έναρξη και στις 4 εβδομάδες για όλους τους ασθενείς και μόνο κατά την έξοδο για την ομάδα AHF.

Αποτελέσματα: Οι τιμές των βιοδεικτών στο σάλιο ήταν σημαντικά χαμηλότερες σε σύγκριση με τις τιμές στον ορό σε όλες τις περιπτώσεις. Μια σημαντική γραμμική συσχέτιση, αν και μέτρια έως ασθενής, μεταξύ του NT-proBNP στον ορό και στο σάλιο βρέθηκε μόνο για ολόκληρο τον πληθυσμό της μελέτης κατά την έναρξη, για τον συνδυασμένο πληθυσμό ΚΑ κατά την έναρξη και για την ομάδα AHF κατά την έναρξη και κατά την έξοδο (όλες είναι ομάδες με υψηλότερες τιμές βιοδεικτών). Ωστόσο, παρουσιάστηκε μια ισχυρή μη γραμμική και πιο σύνθετη σχέση του NT-proBNP στον σάλιο με τον καθιερωμένο βιοδείκτη στον ορό.

Το NT-proBNP στον ορό κατά την έναρξη ήταν σημαντικά υψηλότερο στον συνδυασμένο πληθυσμό ΚΑ (όλοι οι ασθενείς με ΚΑ) σε σύγκριση με τους ασθενείς χωρίς ΚΑ (παχύσαρκοι και υπερτασικοί σε συνδυασμό), τόσο κατά την έναρξη όσο και στον 1 μήνα, και στην AHF σε σύγκριση με τους ασθενείς με CHF. Και στις δύο ομάδες, τα επίπεδα ήταν σημαντικά υψηλότερα σε σύγκριση είτε με παχύσαρκα είτε με υπερτασικά άτομα.

Τα επίπεδα NT-proBNP στο σίελο ήταν σημαντικά υψηλότερα κατά την έναρξη σε ολόκληρο τον πληθυσμό με ΚΑ σε σύγκριση με τον πληθυσμό χωρίς ΚΑ και σε ασθενείς

με AHF σε σύγκριση με τους υπερτασικούς ή τον συνδυασμό των ασθενών χωρίς ΚΑ, αλλά όχι σε σύγκριση με τους ασθενείς με ΚΑ.

Σε ασθενείς με AHF, τα επίπεδα NT-proBNP στον ορό μειώθηκαν σημαντικά από την εισαγωγή έως το εξιτήριο. Το NT-proBNP στον σίελο έδειξε αριθμητική μείωση, αλλά δεν επιτεύχθηκε στατιστική σημαντικότητα.

Τα επίπεδα NT-proBNP στον ορό συσχετίστηκαν με διάφορα κλινικά χαρακτηριστικά σε ασθενείς με ΚΑ, αντανακλώντας τη σοβαρότητα της νόσου όπως αναμενόταν, ενώ οι συσχετίσεις NT-proBNP στο σίελο ήταν σαφώς διαφορετικές και πιο δύσκολο να εξηγηθούν.

Όσον αφορά την κορτιζόλη, τα επίπεδα στον ορό κατά την έναρξη ήταν σημαντικά υψηλότερα στην ΚΑ έναντι όλων των άλλων ομάδων και στον πληθυσμό χωρίς ΚΑ σε σύγκριση με τον πληθυσμό χωρίς ΚΑ. Διαπιστώθηκε ισχυρή συσχέτιση μεταξύ της κορτιζόλης ορού και του NT-proBNP στον ορό, σε ασθενείς με ΚΑ κατά την έναρξη. Δεν βρέθηκε συσχέτιση μεταξύ των επιπέδων κορτιζόλης και NT-proBNP στο σάλιο και μεταξύ της κορτιζόλης στον ορό και στο σάλιο. Δεν βρέθηκαν σημαντικές διαφορές για τις τιμές κορτιζόλης στους σιελογόνους μεταξύ ομάδων και χρονικών σημείων. **Συμπεράσματα:** Τα αποτελέσματα αυτής της μελέτης δείχνουν ότι η κλινική αξία των βιοδεικτών στο σίελο μπορεί να είναι περιορισμένη. Η κορτιζόλη στο σάλιο δεν είχε διαγνωστική, προγνωστική ή παρακολουθητική αξία στην οξεία καρδιακή ανεπάρκεια (AHF) ή τη συμφορητική καρδιακή ανεπάρκεια (CHF), ενώ το NT-proBNP στο σάλιο βρέθηκε να έχει περιορισμένο ρόλο μόνο στη διάγνωση της AHF από έναν γενικό πληθυσμό χωρίς ΚΑ. Δεδομένης της αριθμητικής μείωσης με την ενδονοσοκομειακή περίθαλψη και της συσχέτισης μεταξύ των επιπέδων NT-proBNP στο σάλιο και του ορού σε ασθενείς με AHF, το NT-proBNP στο σάλιο θα μπορούσε να αναζητηθεί στο μέλλον ως ένας πιθανός πολλά υποσχόμενος δείκτης για την παρακολούθηση των ασθενών με AHF και την αξιολόγηση της επίδρασης των παρεμβάσεων. Η μέτρηση βιοδεικτών στο σάλιο, αν και φαίνεται βολική για τους ασθενείς, αποδείχθηκε τεχνικά πολύ δύσκολη, δεδομένης της αφυδάτωσης που προκαλείται από φάρμακα σε αυτούς τους ασθενείς και της περιορισμένης παραγωγής σάλιου. Απαιτείται περαιτέρω έρευνα με μεγαλύτερο μέγεθος δείγματος και βελτιωμένες μεθόδους συλλογής σάλιου και μέτρησης βιοδεικτών, για να διερευνηθεί ο ρόλος του NT-proBNP στο σάλιο στη διάγνωση της ΚΑ και στην ανταπόκριση στη θεραπεία.

ABSTRACT

Objectives: Specific biomarkers in blood have been established as diagnostic and prognostic markers in patients with heart failure (HF); the most important and widely used are the natriuretic peptides, NT-proBNP and BNP. In this thesis, we aimed to investigate the role of salivary biomarkers (primarily NT-proBNP and secondarily cortisol) in the management of acute and chronic HF (diagnosis, risk stratification and response to treatment). The correlation of salivary and serum biomarkers was also investigated.

Methods: Ninety-seven subjects were enrolled; 36 obese or hypertensive with dyspnoea but without a diagnosis of HF (no echocardiographic signs of HF or raised serum NT-proBNP), who served as controls, 12 chronic HF (CHF) patients, and 49 patients with acute HF (AHF – de novo or decompensated) requiring hospital admission. Several clinical, imaging and laboratory data were recorded in all participants at the following timepoints: at baseline and at 4 weeks for all patients and at discharge only for the AHF group.

Results: The saliva values of the biomarkers were significantly lower compared to the serum values in all cases. A significant linear correlation, albeit moderate to weak, between serum and salivary NT-proBNP was found only for the whole study population at baseline, for the combined HF population at baseline and for the AHF group at baseline and at discharge (all are groups with higher biomarker values). However, a strong non-linear and more complex relationship of the salivary NT-proBNP with the established serum biomarker was shown.

Serum NT-proBNP at baseline was significantly higher in the combined HF population (all HF patients) compared to non-HF patients (obese and hypertensive combined), both at baseline and at 1 month, and in AHF compared to CHF patients; in both groups, levels were significantly higher compared to either obese or hypertensive subjects.

Salivary NT-proBNP levels were significantly higher at baseline in the whole HF compared to the non-HF population and in AHF patients compared to hypertensives or the combined non-HF patients, but not compared to CHF patients.

In AHF patients, serum NT-proBNP levels decreased significantly from admission to discharge; saliva NT-proBNP showed a numerical decrease, but statistical significance was not reached.

Serum NT-proBNP levels correlated with various clinical characteristics in HF patients, reflecting disease severity as expected, while the salivary NT-proBNP associations were

distincty different and more difficult to explain.

Regarding cortisol, serum levels at baseline were significantly higher in AHF vs all other groups and in the HF compared to the non-HF population. A strong correlation was found between serum cortisol and serum NT-proBNP, in CHF patients at baseline. No correlation was found between cortisol and NT-proBNP levels in saliva and between serum and saliva cortisol. No significant differences were found for salivary cortisol values among groups and timepoints.

Conclusions: The results of this study indicate that the clinical value of salivary biomarkers may be limited. Saliva cortisol had no diagnostic, prognostic or monitoring value in AHF or CHF, while salivary NT-proBNP was found to have a limited role only in diagnosing AHF from a general non-HF population. Given the numerical decrease with in-hospital treatment and the association between saliva and serum NT-proBNP levels in AHF patients, salivary NT-proBNP could be sought in the future as a potential promising marker for monitoring AHF patients and assessing the effect of interventions. Measuring biomarkers in saliva, although it appears to be convenient for patients, proved to be technically very challenging, given the medication-induced dehydration of these patients and their limited saliva production. Further research with larger sample size and improved methods of saliva collection and biomarker measurement, is needed to explore the role of saliva NT-proBNP in HF diagnosis and response to treatment.

Keywords: acute heart failure; chronic heart failure; heart failure diagnosis; saliva NT-proBNP; salivary biomarkers.

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ACRONYMS - ABBREVIATIONS

6MWT – six minute walk test

8-isoPGF2α - 8-isoprostaglandin F2α

8-epiPGF2α - 8-epiprostaglandin F2α

ACS – acute coronary syndrome

ADHF – acutely decompensated heart failure

AF – Atrial fibrillation

AHF – Acute heart failure

AUC – area under the curve

BNP - B-type natriuretic peptide

CAD – Coronary artery disease

CHF – Chronic heart failure

CI – Confidence interval

CKD – Chronic kidney disease

CMP - Cardiomyopathy

CPET – cardiopulmonary exercise testing

CRP - C-reactive protein

CVD – Cardiovascular disease

DM – Diabetes mellitus

ESC – European Society of Cardiology

Gal-3 - Galectin-3

GDF-15 - Growth differentiation factor-15

HF – Heart Failure

HFmrEF – Heart failure with mid-range ejection fraction

HFpEF - Heart failure with preserved ejection fraction

HFrEF - Heart failure with reduced ejection fraction

HR – Hazard risk

hsTn - High sensitivity troponin

IL-6 interleukin 6

IL-10 interleukin 10

LOD - Limit of detection

LVEF – Left Ventricular Ejection Fraction

LV - Left ventricle/ left ventricular

MI – Myocardial Infarction

NT-proBNP - N-terminal pro-B type natriuretic peptide

NYHA - New York Heart Association

OR – odds ratio

PAWP - pulmonary arterial wedge pressure

RAP – right atrial pressure

RR – relative risk

RV – Right ventricle/ right ventricular

sAA - Salivary alpha amylase

SCD – Sudden cardiac death

SD – Standard deviation

S100A7 -Protein S100-A7

ST2 - Soluble interleukin 1 receptor-like 1

TNF-α - Tumor necrosis factor-α

TR – Tricudpid valve regurgitation

UA - Uric acid

GENERAL PART:

NOVEL BIOMARKERS IN HEART FAILURE

INTRODUCTION

DEFINITION OF HEART FAILURE (HF)

Heart failure (HF) is a clinical syndrome characterized by typical symptoms (such as dyspnoea, breathlessness, orthopnea, paroxysmal nocturnal dyspnoea, reduced exercise tolerance) and signs such as elevated jugular venous pressure, third heart sound, pulmonary or peripheral congestion (pulmonary crackles, ascites, pleural effusion, bilateral oedema). It is caused by structural and/or functional cardiac abnormalities that result in a reduced cardiac output and/ or elevated intracardiac pressures at rest or during stress (1). The importance of the term «syndrome» lies on the fact that a person may have a structural and/or functional cardiac abnormality (e.g. reduced left ventricular ejection fraction –LVEF) without having symptoms. In that case this abnormality (e.g. systolic dysfunction) is a precursor and only when symptoms occur, the diagnosis of HF is posed (1). The precursor stage is important because it is related to poor outcomes, while starting treatment early may reduce mortality in patients with asymptomatic systolic or diastolic LV dysfunction (2).

The etiology of HF is quite variable, with most common causes being coronary artery disease, hypertension, arrhythmias, valvular and structural abnormalities, toxic damage from recreational substance use and chemotherapy, immune, inflammatory and metabolic causes, infiltrative diseases, genetic abnormalities, etc. (3). Many patients may have more than one causative factor for HF. The etiology of HF is presented in **Table 1**.

Table 1: Etiology of Heart Failure	
Myocardial Diseases	
Ischemic heart disease	
Toxic damage	Recreational substance use, heavy metals, medications, radiation
Infectious diseases	Bacteria, spirochaetes, fungi, protozoa, parasites (Chagas disease), rickettsiae, viruses (HIV/AIDS).
Inflammatory -	Lymphocytic/giant cell myocarditis, Graves' disease,

autoimmune diseases	rheumatoid arthritis, connective tissue diseases, eosinophilic myocarditis (Churg–Strauss).
Infiltrative diseases	Amyloidosis, sarcoidosis, haemochromatosis, glycogen storage diseases, lysosomal storage diseases.
Malignancies	Primary or metastatic
Genetic cardiomyopathies	Hypertrophic, dilated, non-compaction, arrhythmiogenic, restrictive, muscular dystrophies, laminopathies.
Metabolic diseases	Thyroid and parathyroid diseases, hypercortisolemia, Conn's disease, Addison disease, diabetes, metabolic syndrome, phaeochromocytoma, pathologies related to pregnancy and peripartum.
Nutritional disorders	Deficiency of thiamine, L-carnitine, selenium, iron, phosphates, calcium.
Abnormal Loading Conditions	
Hypertension	
Valvular diseases	
Structural defects	Atrial and ventricular septum defects
Pericardial diseases	Constrictive pericarditis, pericardial effusion
Endocardial diseases	Hypereosinophilic syndrome, endomyocardial fibrosis, endocardial fibroelastosis
Volume overload	Renal failure, self-induced water intoxication, iatrogenic fluid overload
High output conditions	Anemia, sepsis, thyrotoxicosis, arteriovenous fistula, pregnancy, Paget's disease.
Arrhythmias	
Bradyarrhytmias	Sinus node dysfunction, conduction disorders
Tachyarrhytmias	Ventricular, atrial arrhytmias

Furthermore, many pathophysiological mechanisms are primarily or secondarily implicated in HF: myocardial fibrosis, inflammation, intermediary

metabolism, adipocyte biology and angiogenesis, mineral metabolism/calcification, liver fibrosis etc. and a systematic approach is necessary to fully comprehend its clinical course and prognosis.

In clinical practice, HF classification for the management of patients is based on LVEF, rather than the etiology. Accordingly, patients are characterized as HF with preserved, mid-range (or mildly reduced) and reduced LVEF (HFpEF, HFmrEF and HFrEF when LVEF is ≥50%, 40–49%, and <40%, respectively) (3). Until recently it was believed that the majority of HF patients belong to HFrEF, however large cohort studies revealed that up to 50% of the HF population belong to HFpEF group, with equally poor outcome. Unlike HFrEF, there is no demonstrated beneficial therapy for HFpEF in terms of hospitalizations and mortality (4), at least not until recently. This may be partially related to the poor understanding of the pathophysiological pathways of HFpEF. In addition to the causative uncertainty, the diagnosis and risk stratification of these patients also represent critically unmet needs (5).

Patients with new onset HF are described as de novo HF patients, while if they have been diagnosed with HF for some time they are considered chronic HF patients. If patients with chronic HF have unchanged symptoms and signs for at least 1 month, they are considered stable. When chronic stable HF patients deteriorate, they are described as (acutely) decompensated.

In order to describe symptoms, the most common classification is the New York Heart Association (NYHA) functional classification which has four stages (NYHA I to NYHA IV), although a patient can be rendered asymptomatic by treatment. Advanced HF refers to patients that remain severely symptomatic despite optimal guideline-directed management, regardless of LVEF, including patients who remain ambulatory but are essentially NYHA-III (Advanced) to IV and are characterized by a) severe cardiac dysfunction defined by systolic or diastolic LV dysfunction, isolated RV failure, non-operable severe valve disease, congenital abnormalities or persistently high (or increasing) BNP or NT-proBNP values, b) episodes of pulmonary or systemic congestion requiring high-dose intravenous diuretics or diuretic combinations c) episodes of low output requiring inotropes or vasoactive drugs or malignant arrhythmias causing more than one unplanned visit or hospitalization in the last 12 months d) severe impairment of exercise capacity with inability to exercise or low six

minute walk test (6MWT) distance less than 300 meters or pVO2 less than 12–14 mL/kg/min in cardiopulmonary exercise testing (CPET), estimated to be of cardiac origin e) extra-cardiac organ dysfunction due to heart failure (6).

At the end of the HF spectrum lays the cardiogenic shock, which is also a clinical syndrome and it's diagnosis requires the presence of clinical signs of hypoperfusion (cold sweated extremities, oliguria, confusion, dizziness, narrow pulse pressure), and biochemical manifestations of hypoperfusion, elevated creatinine, metabolic acidosis and elevated serum lactate, while haemodynamic parameters, such as reduced cardiac index (CI) and/or elevated pulmonary capillary wedge pressure (PCWP), may not be necessary (7).

Approximately 64.3 million people are diagnosed with HF worldwide (8) accounting for 9.91 million years lost due to disability (YLDs) and 346.17 billion US dollars expenditure (9). In developed countries, the prevalence is estimated between 1-2% of the adult population; in the USA the HF percentage is estimated at 2.5%, in the UK at least 1.6%, while in Germany it reaches 4% (10). Moreover, half of the patients having a preserved ejection fraction and it is expected that this proportion while increase in the proximate future (10). Interestingly a meta-analysis of studies using echocardiographic screening criteria in the general population raised further the prevalence of all type HF to 11.8% in those aged 65years and over in the developed countries, which would account for a calculated prevalence of 4.2% in the general population (11) which is rather a more realistic estimate. The fact is that the number of HF patients will keep increasing, given the population growth, the aging population and the improved survival after initial diagnosis. According to estimations based on the third-degree polynomial equations, prevalence and YLDs will probably grow to 9.81 per 1,000 inhabitants (+15.1%) and 12.82 per 1,000 YLDs (+10.4%) by the year 2030, accounting for approximately 398.44 billion US dollars worldwide expenditure (9).

The incidence of HF in European countries and the USA ranges from 1 to 9 cases per 1000 person-years and strongly depends on the population studied and the diagnostic criteria that are used (10). A study with 1.25 million individuals from the UK showed that the overall chance of a 30-year-old person to develop HF during

the rest of his or her life is 5% and in hypertensive persons this percentage rises to 7.8% (12).

Concerning the sex differences, women have a significantly lower incidence rate of HF compared to men (at all age categories except >74 years) (13), but they still account for approximately half of the prevalent cases since HFpEF may be more common in women (10) although this was not proved in the Framingham Heart Study, in which female sex was not associated with an increased risk of HFpEF, but with a lower risk of HFrEF (14). Females are on average up to 5 years older at first HF diagnosis compared to males (79.6 vs. 74.8 years), with a lower median survival (3.99 vs. 4.47 years), but they have a 14% age-adjusted lower risk of all-cause death [hazard ratio (HR) 0.86, 95% confidence interval (CI) 0.84–0.88] (15). Another characteristic is that quideline-directed medical therapy tends to be less intense over time in women compared to men (16). Futhermore, females are more often obese than males and the relationship between obesity and incident HFpEF seems stronger in women (17). Finally, a recent meta-analysis of 47 cohort studies, including 12 million individuals, found that the relative risks for HF associated with type 2 diabetes were 1.95 (95% CI 1.70–2.22) in women and 1.74 (95% CI 1.55–1.95) in men, with a pooled men-to-women ratio of the relative risks of 1.09 (95% CI 1.05–1.13), indicating a significant difference (18).

HF DIAGNOSIS

The diagnosis of HF requires the presence of symptoms and/or signs but also objective evidence of cardiac dysfunction, since symptoms and signs alone lack sufficient accuracy to pose the diagnosis of HF (4). Typical symptoms of HF include breathlessness, orthopnoea, paroxysmal nocturnal dyspnoea, fatigue, reduced exercise tolerance, and ankle swelling. Specific signs are an elevated jugular venous pressure, the hepatojugular reflux, a third heart sound (gallop rhythm), or a laterally displaced apical impulse. Less specific signs are abnormal weight gain (>2 kg/week) or loss (seen in advanced HF, cachexia), peripheral oedema (ankle, sacral, scrotal), pulmonary crepitations, pleural effusions, tachycardia, hepatomegaly, ascites, oliguria, and narrow pulse pressure (1, 8).

According to the latest guidelines on HF by the European Society of Cardiology (ESC), the diagnostic algorithm for the diagnosis of HF in a patient with symptoms and signs of HF and risk factors for HF (such as a history of myocardial infarction -MI, arterial hypertension, coronary artery disease - CAD, diabetes, alcohol misuse, chronic kidney disease - CKD, chemotherapy, and family history of cardiomyopathies – CMP or sudden cardiac death – SCD) starts with an abnormal electrocardiogram (ECG). The ECG may reveal abnormalities like atrial fibrillation (AF), signs of current or previous ischemia, LV hypertrophy (LVH), and a widened QRS complex. An abnormal ECG has 89% sensitivity for diagnosing HF (19).

Natriuretic peptides (i.e. BNP and/or NT-proBNP) are the only biomarkers that have an established role in the diagnosis of HF and when used in addition to standard clinical investigations, were either dominant (less costly and more effective) or cost-effective across different countries (20). If the natriuretic peptides are raised (BNP>35pg/mL, NT-proBNP>125 pg/ml) or unavailable, they lead to an echocardiogram.

If there are abnormal findings in the echocardiogram (dilated or hypertrophic cardiac chambers, regional wall motion abnormalities, reduced RV function, pulmonary hypertension, valvular dysfunction, or diastolic dysfunction) then the diagnosis of HF is posed, otherwise other diagnosis should be considered (4).

Apart from those tests other basic investigations such as full blood count, serum urea and creatinine, electrolytes, liver and thyroid function, fasting glucose and hemoglobin A_1C, lipidemic profile and iron status are generally used to differentiate HF, guide treatment and stratify patients.

A chest X-ray if available may also assist in revealing other reasons of dyspnoea (lower respiratory tract infection, pneumothorax, emphysema) or provide supportive evidence of HF (e.g. increased cardiac size or pulmonary congestion).

HF THERAPY

Although the three HF categories (i.e. HFrEF, HFmrEF and HFpeEF) have little or no differences in etiology, risk stratification and prognosis, the therapeutic strategies are perhaps the area where they vary the most. The common goal of treatment is to improve the clinical condition of patients and reduce hospitalizations, adverse events and mortality, reduce the direct and indirect costs and improve quality of life for all HF spectrums (21).

Among HF groups, HFrEF is the one that has the most medication categories studied with numerous randomized clinical trials (RCTs), while pharmacotherapy is the cornerstone prior to device therapy. Down-regulation of the renin-angiotensin-aldosterone system (RAAS) and sympathetic nervous system (SNS) with angiotensin-converting enzyme inhibitors (ACE-I) or angiotensin receptor-neprilysin inhibitors (ARNI), beta-blockers, and mineralocorticoid receptor antagonists (MRA) has been shown to improve survival, reduce the risk of HF hospitalizations, and reduce symptoms (1). SGLT2 inhibitors (i.e. empagliflozin and dapagliflozin) increased the benefit when added to therapy with the three above mentioned categories as they further reduced the risk of CV death and worsening HF in patients with HFrEF, regardless of the presence of diabetes mellitus (DM) (22, 23). Diuretics and especially furosemide is also first class medication for signs and/or symptoms of congestion in order to reduce symptoms, improve exercise capacity, and reduce HF hospitalizations (4). Ivabradine should be used in symptomatic patients with LVEF ≤35%, in sinus rhythm and resting heart rate of more than 70 bpm, who are unable to receive a beta-blocker, to reduce the risk of HF hospitalization and cardiac death (4), while hydralazine and isosorbide dinitrate should be considered in NYHA III-IV patients with dilated LV (4). Furthermore, implantable devices are used in patients already on optimal medical treatment for three months (unless when used for secondary prevention) (24). Implantable cardioverter-defibrillators (ICD) are used to reduce sudden cardiac death (SCD) and all-cause mortality (25), while cardiac resynchronization therapy (CRT) is used in order to improve symptoms and reduce morbidity and mortality (26, 27).

In HFmrEF, diuretics should be used to control congestion similarly to HFrEF. However, there are no prospective RCTs exclusively with HFmrEF patients, and there are no medications that their use has met any primary endpoint of all-cause mortality or HF hospitalizations. Implantable devices for SCD prevention or resynchronization therapy are also not recommended (4).

Similarly to HFmrEF, no treatment has been shown to convincingly reduce mortality and morbidity in patients with HFpEF and the treatment relies mostly in the predominant risk factors (4). For instance, most HFpEF patients suffer from hypertension and/or CAD, so medications for these diseases (like ACE-I/ARBs, beta-blockers, or MRAs) are already being used. Once again, diuretics are used to control symptoms and signs of congestion.

Finally, for all HF categories, etiologic therapy should be implemented for structural anomalies. For instance, either surgical (SAVR) or transcatheter (TAVR) aortic valve replacement should take place in aortic stenosis, depending on the risk and patients' profile to reduce mortality and improve symptoms (28, 29). Likewise, mitraclip should be considered for secondary mitral regurgitation etc. (30, 31).

HF PROGNOSIS

Although the prognosis of HF has improved over the last decades, it still remains poor, while this trend levels off (32). The quality of life (QoL) is also low (4). The overall mortality rates one year and five year after diagnosis for all types of HF is 20% and 53% (33), although the five-year mortality in HFrEF is reported to be up to 67% (34). Women with HF are frequently prescribed with fewer categories of medications and in lower doses compared to men (35); despite that less evidence-based treatment they have a lower one-year mortality than men (36) and lower rates of all-cause-hospitalization, without any differences in the causes of death (37). When comparing the various HF categories, a large meta-analysis reported lower adjusted mortality risk in HFpEF compared to HFrEF (38). Furthermore, one-year outcomes in HFmrEF are also better than HFrEF (39).

After initial diagnosis, every patient with HF is expected to have one hospitalization per year, whether it is HF related or not. Recent data show a trend for increase in hospitalizations, especially for women and especially for non HF-related reasons (42% versus 28%) (40). The risk of HF hospitalization is 50% higher in patients with diabetes compared to controls. Other risk factors that increase HF hospitalizations are obesity, renal impairment and the presence of AF (4).

In patients admitted with acute HF, whether acute decompensated or de novo HF the in hospital mortality reached 5.3% (41). The highest rate was noted in ‘wet-cold’ patients (12.1%) vs. 9.1%, 2.0% and 3.8% in the ‘dry-cold’, ‘wet-warm’ and ‘dry-warm’ categories, respectively (41). After one-year follow up the all-cause mortality ranged from 12.1% in ‘dry-warm’ to 26.4% in ‘wet-cold’ patients, while most of deaths were due to cardiovascular causes (41). The AHF patients that presented wet (whether warm or cold) had the highest one-year HF-related hospitalization rate (41). As expected, patients with residual congestion on discharge had higher one-year mortality compared with patients without congestion on discharge (41).

With an ageing population globally the prevalence of HF is expected to rise dramatically, up to 50% (42). This will unavoidably lead to an increase in hospitalizations and health expenditure as well.

BIOMARKERS

Role in clinical practice

According to the Biomarkers Definitions Working Group (NIH Definitions Working Group, 2000) convened by the U.S. National Institutes of Health (NIH) a biomarker is a characteristic that is objectively measured and evaluated as an indication of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (43). According to a more recent definition, a biomarker is a functional variant or quantitative index of a biological process that predicts or reflects the evolution of or predisposition to a disease or a response to therapy (43). Categories of biomarkers include: susceptibility/risk biomarkers, diagnostic biomarkers (used to identify or confirm the presence of a disease or condition), monitoring biomarkers, prognostic biomarker, predictive biomarkers, pharmacodynamic/response biomarkers and safety biomarkers (44).

Clinicians use biomarkers for the diagnosis, prognosis and therapy monitoring of various diseases. In the context of precision medicine, biomarkers enable two forms of enrichment to recruit patients for future clinical trials in critical care; the prognostic enrichment, which discriminates patients at increased risk of an outcome of interest, and the predictive enrichment which predicts which patients are more likely to respond to a specific treatment based on their specific clinical and/or biological phenotype (45).

A biomarker in medicine can be a bio-sample (blood, urine, tissue), but it may also be a recording such as an electrocardiogram (ECG), or an imaging test (echocardiogram, magnetic resonance imaging [MRI], or computed tomography [CT] scan) (46). Genomic variations, mainly single nucleotide polymorphisms (SNPs), are dominant markers of the genetic variability in humans, and a multitude of genomics studies have emerged as novel biomarker candidates. Other possible biomarkers are non-coding RNAs (ncRNAs), which have been assessed in a range of diseases, including CVD (47). The best-studied ncRNA species are microRNAs (miRNAs), defined by the small number of nucleotides (18–25) of which they are comprised, since they are protected from degradation and therefore are stably detectable in the circulation (48). Furthermore, the combination of multiple individual biomarkers in a

score has gained grounds as likely to improve sensitivity and discriminate among healthy subjects and low-risk patients. Saliva is a biological fluid that is also gaining ground as a potential bio-sample. It contains several proteins that can be detected and could potentially be used as biomarkers, while the fact that it can be easily collected with non-invasive procedures is a major benefit compared to blood (3). On top of that salivary diagnostics is currently catching onto the emerging field of Lab-on-Chip (LoC) and Point-of-Care (PoC) devices (3).

When evaluating bio-molecules for their potential to serve as disease biomarkers, several criteria need to be fulfilled, such as the sensitivity and specificity, detectability and stability, accessibility in routine procedures, comparability of measuring techniques, the reference values, the pathophysiological soundness and differential quantification between diseased and healthy controls, the diagnostic and/or prognostic validation, the additional value it adds to the current standard clinical evaluation and risk stratification and others (41).

The greatest advantage of a biomarker should be the fact that it should be cheaper and easier to measure than final clinical endpoints while its measurement should be reproducible over time. Disadvantages may be the storage (longevity of samples), the measurement technique and laboratory errors, the time needed for measurement which may be critical, the difficulty in establishing normal range, the ethical responsibility and others (49).

So far several biomarkers have been used in HF management, either plasma biomarkers (**Table 2**), or in saliva (**Table 3**) and urine (**Table 4**). In the next chapter the most-well studied and widely used will be described.

BLOOD BIOMARKERS

1. Natriuretic Peptides (NPs)

a) Pathophysiology and role in HF Diagnosis

Natriuretic peptides (NPs) include three main forms: i) atrial natriuretic peptide (ANP), ii) B-type natriuretic peptide (BNP), and iii) C-type natriuretic peptide (CNP) (50). Synthesis of NPs is mediated by the increased cardiac wall stress (Fig 1.) (51).

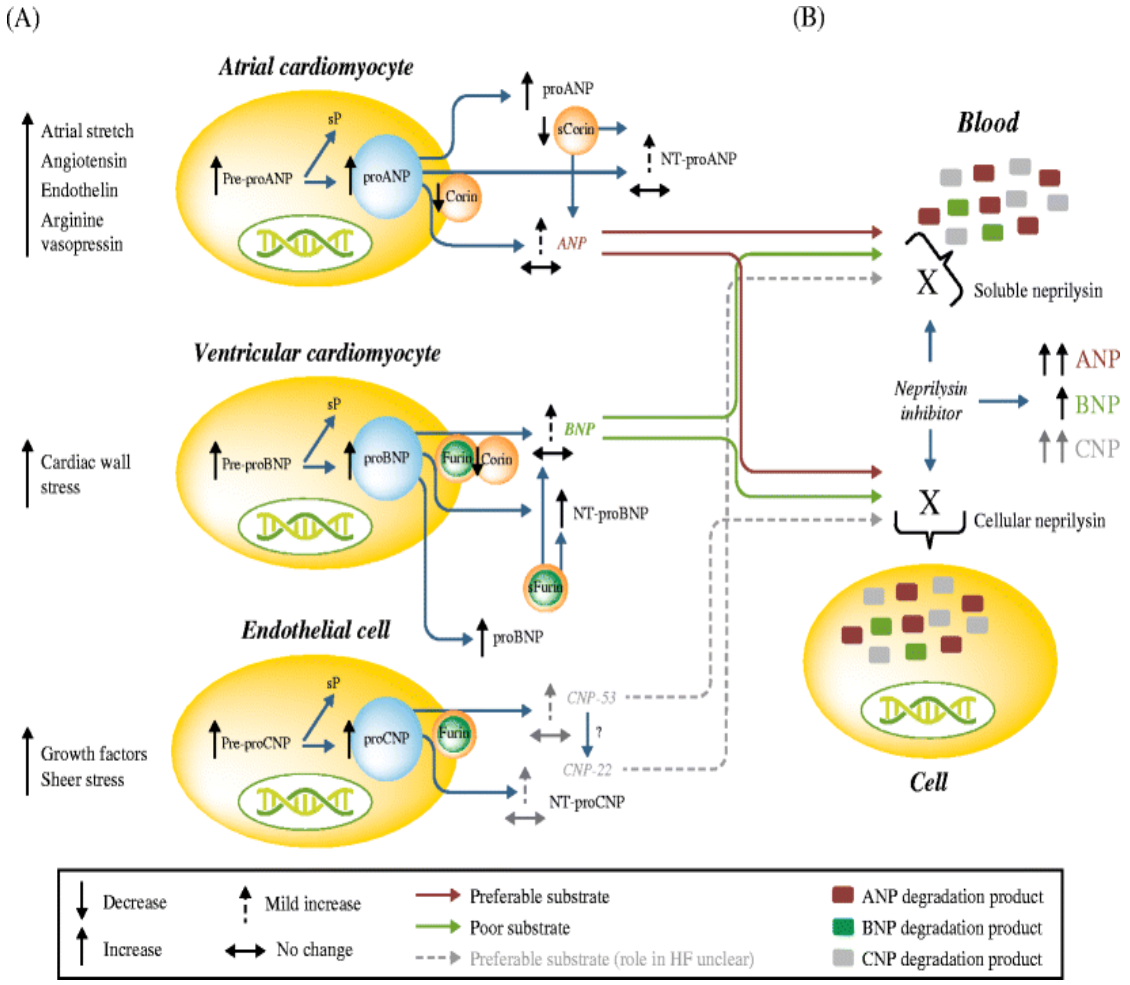


Figure 1. *Synthesis and processing of NPs via neprilysin in patients with HF* ***a)*** *ANP is synthesized in atrial cardiomyocytes as pre-proANP; the sP of which is cleaved to form proANP. Upon secretion, proANP is processed by membrane-bound and soluble corin, generating an N-terminal peptide (NTproANP; 98 amino acids), and an active C-terminal peptide (ANP; 28 amino acids). In patients with HF, corin levels are decreased, resulting in an increase in predominantly unprocessed ANP. BNP is*

synthesized as pre-proBNP in ventricular cardiomyocytes. Removal of a sP from pre-proBNP forms proBNP, which is processed by membrane-bound and soluble furin, and corin, to release the N-terminal portion (NT-proBNP; 76 amino acids), and the biologically active C-terminal (BNP; 32 amino acids). CNP is widely expressed in the vasculature and found in high concentrations in the endothelium. CNP expression has also been reported in cardiomyocytes at gene and protein levels. CNP is synthesized as pre-proCNP; the sP of which is cleaved to form proCNP. Processing of proCNP (103 amino acids) may occur via furin to yield a53 amino acid C-terminal peptide (CNP-53), the major active form of CNP in the tissues. In the systemic circulation, a 22 amino acid form of CNP dominates (CNP-22), but the protease responsible for this cleavage is unknown. ***b)*** *Cellular and circulating soluble neprilysin are major contributors to ANP degradation. In contrast, BNP is a poor neprilysin substrate. Thus, neprilysin inhibition (e.g., via sacubitril) is most likely associated with greater augmentation of ANP activity than BNP activity. Neprilysin also has a high affinity for CNP, and as such, inhibition of neprilysin is expected to increase levels of CNP. ANP atrial natriuretic peptide, BNP B-type natriuretic peptide, CNP C-type natriuretic peptide, HF heart failure, NP natriuretic peptide, NTproANP N-terminal proANP, NT-proBNP N-terminal proBNP, NTproCNP N-terminal proCNP, sP signal peptide, TGF transforming growth factor [51].*

ANP is released primarily by the atrial myocardium in response to dilatation; BNP by the ventricular myocardium in response to elevations of end-diastolic pressure and volume, while CNP is released by endothelial cells in response to shear stress and can also eliminate NPs from the circulation to reduce plasma concentrations (52, 53). Both ANP and BNP are synthesised as aminoacid precursor proteins that are intracellularly modified to prohormones. Pro-ANP is cleaved into a 98 aminoacid N-terminal fragment (N-terminal ANP) and then the 28 aminoacid active hormone (ANP) is released into the circulation (52). It has been suggested that proBNP is cleaved into active BNP molecule and the inactive marker molecule (N-terminal proBNP, NT-proBNP) intracellularly by processing enzyme furin, and they are released into the circulation (54). NT-proBNP is a 76 aminoacid N-terminal fragment, synthesised and released from ventricular myocytes, while the active hormone (BNP) consists of 32 aminoacids (55). Recent studies have shown that considerable amount of uncleaved proBNP circulates in the blood, and the commercially available BNP assay kits consist of two antibodies that sandwich the

BNP molecule (54). Therefore, if proBNP is present, BNP assay kit cross-reacts to proBNP and measures it as BNP (54).

NPs relax vascular smooth muscle which causes vessel dilation and leads to reduced blood pressure and ventricular preload (52). BNP has a suppressive function on SERCA2a and S100A1, two Ca^{2+}-regulatory proteins that significantly affect systolic and diastolic function (50). Moreover, BNP activates natriuresis, diuresis, and inhibits the renin–angiotensin–aldosterone axis and sympathetic activity in response to ventricular dilatation and pressure overload [***Fig 2*** (56)].

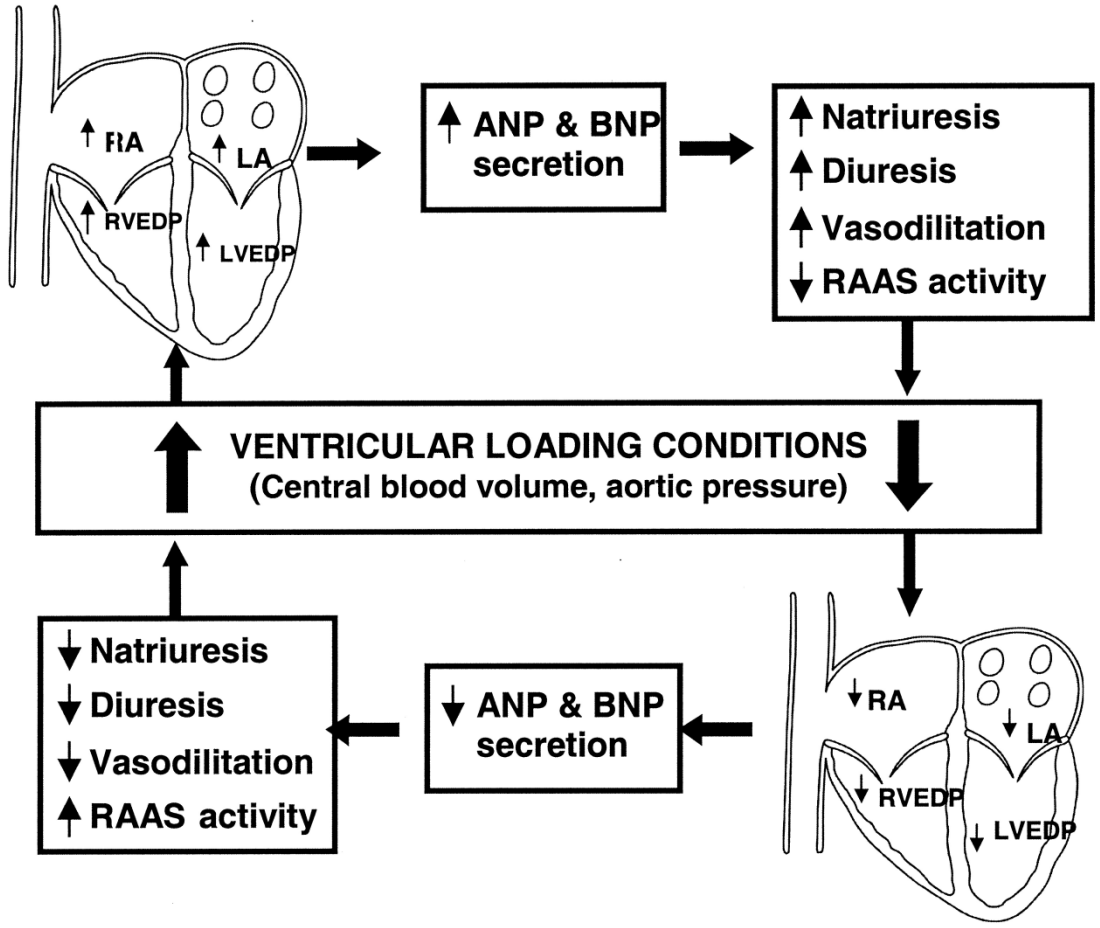


Figure 2*. Schematic diagram depicting the mechanism by which cardiac secretion of natriuretic peptides serves to maintain intravascular volume homeostasis. ANP = atrial natriuretic peptide; BNP = B-type natriuretic peptide; LA = left atrium; RA = right atrium; LVEDP = left ventricular end-diastolic pressure; RAAS = renin-angiotensin-aldosterone system; RVEDP = right ventricular end-diastolic pressure.*

In HF, all three NPs increase but BNP increases more rapidly (57, 58). Despite incremental increases in circulating NP concentrations with HF progression, their effects are attenuated and several major factors may account for that. There is reduced availability of active forms of NPs, namely BNP, secondly target organ responsiveness becomes diminished and finally the counter-regulatory hormones of the RAAS and SNS, and endothelin-1 become over-activated (59). In a recent study plasma proBNP and total BNP (proBNP+mature BNP) were measured in AHF patients (n=154) on admission, day 3, day 7, and on discharge, and the percent proBNP was calculated as: (proBNP/total BNP) × 100 (60). On admission, %proBNP was higher in patients with severe AHF than in patients with mild AHF (median: 61.7% vs. 56.2%, respectively; $p<0.01$), while the plasma cGMP/total BNP ratio, an index of the biological activity of BNP, was lower ($p<0.001$) (60). In severe AHF, the higher %proBNP and lower cGMP/total BNP ratio were not changed during hospitalization, while in mild cases the %proBNP increased gradually and the cGMP/total BNP ratio increased on days 3, suggesting that in mild AHF, compensation for HF occurs via increased proBNP processing, leading to increase of mature BNP and activation of the BNP/cGMP cascade while in severe AHF this mechanism may be impaired (60).

In early pilot studies, increased BNP levels distinguished HF from other causes of dyspnoea more accurately than LVEF, ANP, and N-terminal ANP, with sensitivity greater than 90% and specificity of 80–90% (61). These findings were afterwards supported a series of studies and finally BNP and its precursor NT-proBNP are nowadays officially incorporated in the diagnostic algorythm of HF, both in the ESC and AHA guidelines, with a cut-off of 35pg/ml and 125pg/ml respectively in a non-acute setting (4, 62). Raised concentrations of NPs support the diagnosis of HF, are useful for prognostication, and may guide further cardiac investigation (63). Nevertheless there are many cardiac and non-cardiac causes of elevated NPs that might reduce their diagnostic accuracy, like ACS, pulmonary embolism, myocarditis, valvular heart disease, AF, elderly age, acute or chronic kidney disease, ischemic stroke, infections, anemia, hyperthyroidism, etc. (4). In a registry from the ESC-Heart Failure Association (HFA) EURObservational Research Programme (EORP) with hospitalized patients with raised NP values (BNP≥100 pg/mL and/or NT-proBNP ≥300 pg/mL) and LVEF ≥ 50%, classified as HFpEF, only one third of subjects had elevated

LA filling pressures and the others could have been misdiagnosed (or had echocardiography after normalization of LA pressure) (64). These subjects were more often hospitalized for non-HF reasons during follow-up and their symptoms suggestive of acute HFpEF and NP values may stemp from non-HF comorbidities (64). Especially for BNP, use of sacubitril/valsartan, and macro-proBNPemia overestimate its value, whereas the presence of pericardial effusion, or an acute coronary syndrome onset underestimate it (54). NP concentrations may also be disproportionately low in obese patients (65). There is an inverse relationship of NPs with high BMI in HFrEF and HFpEF, with a tendency of higher levels in HFrEF; however the prognostic value of NPs is preserved across BMI classes, and increased circulating levels of NPs correlate with adverse outcomes including all-cause mortality and HF hospitalizations (66). Lower thresholds should be utilized in decision making when it comes to obese subjects and there are still limited data for patients with severe obesity (BMI > 40 kg/m^2) (66). In anemic patients, a retrospective study (n=3.756 patients with or without ischemic heart disease -IHD) found that LVEF increased in response to low haemoglobin (Hb), and the phenomenon was prominent in non-IHD (standardised regression coefficients (St.β): -0.264, $p<0.001$) rather than in IHD (St.β: 0.015, $p=0.531$), but the response of BNP was commonly observed in both groups (non-IHD St.β: -0.238, IHD St.β: -0.398, $p<0.001$, respectively) (67). Finally NT-proBNP levels tend to be lower in black individuals, compared to white and Hispanics (68).

A study with hospitalized AHF patients (n=182, NYHA III-IV; 96%) measured the circulating levels of proBNP, NT-proBNP, and total BNP (proBNP+mature BNP) to correlate their change with worsening renal function (WRF), defined as a >20% decrease in the estimated glomerular filtration rate (eGFR) during hospitalization (69). NT-proBNP did not differ among patients with and without WRF, while mature BNP was lower in patients with WRF on day 3 after admission and NT-proBNP/matureBNP ratios were strongly associated with percentage decreases in eGFR and preceded WRF (69). The NT-proBNP/matureBNP ratio could be used as a biomarker for the prediction of cardiorenal syndrome (69).

b) NPs in HF prognosis and risk stratification

The Acute Decompensated Heart Failure National Registry (ADHERE) that included AHF patients (n=48.629) found a significant association between admission BNP values and intra-hospital mortality, that was independent of other clinical and laboratory parameters (age, gender, SBP, heart rate, dyspnoea, sodium, creatinine, and urea levels and LVEF) (70). Admission NPs (either BNP or NT-proBNP) of 5.625 patients from the Korean AHF registry significantly improved the net reclassification and integrated discrimination for one-year mortality of the Meta-analysis Global Group in Chronic Heart Failure (MAGGIC) score (NRI of 33.4%, $p<0.0001$ and IDI 0.002, $p<0.0001$) which uses 13 routinely available patient characteristics (age, gender, DM, COPD, HF diagnosed within 18 months, current smoker, NYHA class, use of beta blocker, ACEI or ARB, BMI, systolic blood pressure, creatinine, LVEF) (71). The discrimination abilities of the MAGGIC score with and without NPs did not improved significantly (C-index 0.734 without and 0.736 with NPs, $p=0.0502$) (71). Usually either BNP or NT-proBNP is measured; however the plasma levels of the two biomarkers do not always increase proportionally and the combination of testing both might add prognostic value to predict adverse events in hospitalized HF patients (72). In 1207 AHF patients, a high NT-proBNP/BNP ratio measured 15min after admission was associated with the presence of a non-cardiac condition (e.g. inflammatory reaction, nutritional status, and renal dysfunction) and was independently associated with worse 180-day mortality [HR 3.084, 95% CI 1.838-5.175] and HF events (HR 1.963, 95% CI 1.340-2.876) (73).

During hospitalization, BNP reduction in AHF patients (n=1028, age, 77 ± 13 years; 57% male; LVEF 47 ± 16%), defined as [(BNP on admission - BNP at discharge) / BNP on admission × 100] was significantly related to all-cause 1-year mortality (HR 0.96, 95% confidence interval 0.93-0.99, $p=0.032$), while percentage body weight reduction was not, and it was also more predictive in HFrEF than HFpEF (74). In 245 patients from the ESCAPE trial, the relationship between BNP reduction form admission to discharge and several clinical and objective markers of decongestion were studied. Patients were divided into four tertiles according to biomarker

reduction (75). There were significant differences across tertiles with regard to resolution of jugular venous distention (JVD, p=0.014), orthopnea (p=0.04), weight loss (p=0.002), and inferior vena cava (IVC) diameter (p=0.0001) (75). A significant correlation between admission-to-discharge BNP reduction and admission-to-discharge weight loss (n=232, r=0.211, P=0.001), admission-to-discharge reduction in IVC diameter (n=99, r=0.360, P<0.0001) and reduction in PCWP from admission to the day of pulmonary artery catheter removal (n=92, r=0.242, P=0.02) was found (75). In a study with 224 patients (median age 80 years, 42.9% men, 63.9% HFrEF) hospitalized for AHF, from who 112 survived after six months and 112 not, BNP decrease was the only independent mortality predictor in multivariate analysis (76). Patients with a BNP decrease >30% had an HR of death of 0.57 (0.37 to 0.89) in 6 months (76). Other literature suggested predictors of death (AF, hypertension, admission heart rate, SBP, NYHA class, hemoglobin, urea, albumin, systolic dysfunction, ischemic etiology) were dependent of NPs (76).

Three studies that measured BNP on admission, prior to discharge and their change during hospitalization in AHF patients both concluded that discharge BNP is a better prognostic biomarker for long-term mortality. A registry with 7.039 patients with AHF (Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure, OPTIMIZE-HF), reported that discharge BNP is a better predictor of 1-year mortality (HR 1.34, 95% CI 1.28–1.40) and 1-year mortality or hospitalization (HR 1.15 95% CI 1.12–1.18) than admission BNP (77). The second study (n=433, median age 56years, 74% men) where admission BNP, discharge BNP and admission-to-discharge percentage BNP reduction were measured (mean 1009pg/mL, 743pg/mL and -70% respectively), showed that discharge biomarker values had the highest AUC for predicting mortality (AUC=0.702, p<0.001) followed by admission BNP (AUC=0.633, p=0.0006) and then by the percentage BNP reduction (AUC=0.620, p=0.008) (78). Importantly there were significant differences between survivors and nonsurvivors in all time-points (p=0.0001 for admission and discharge, p=0.001 for percentage BNP reduction) (78). Cox-proportional-hazard-analysis revealed that discharge BNP is an independent predictor of 6-month mortality (HR=1.063, 95% CI=1.037-1.089, p<0.001) while the combination of a discharge BNP

≤319pg/mL and percentage BNP reduction ≥7.71% was associated with significantly lower mortality (4.8% vs 27.2%, RR=0.134, 95% CI=0.046-0.387, p<0.001) (78). Similarly in the third study (n=1.792 AHF patients, 860 HFrEFs, 318 HFmrEFs, and 614 HFpEFs), discharge values had the highest c-index (0.69) for mortality (p<0.001) (77). In multivariate Cox proportional hazard modeling, discharge BNP was significantly associated with mortality in all categories (HR 1.95, 95% CI 1.57 to 2.41 for HFrEF; HR 1.76, 95% CI 1.10 to 2.82 for HFmrEF; HR 1.46, 95% CI 1.12 to 1.91 for HFpEF, p=0.011) while the c-index of discharge BNP for mortality was higher in HFrEF (0.72, 0.68 and 0.65 respectively) (79).

An interesting study with AHF patients (n=584, median age 78, 62.5% HFrEF) that had BNP measurement prior to discharge reported that for values lower that 400pg/mL one year mortality depended on other commorbidities like age, while for values higher that 2000pg/mL death predictors were higher NYHA class, and non-use of evidence-based therapy (80). Finally, BNP was outcome-associated in both groups (80).

The discharge BNP enhanced the prognostic performance of the MAGGIC score in a Japanese AHF registry (n= 2.215, mean age 73.0 ± 13.0, 61.2% male) (81). The MAGGIC score demonstrated modest discrimination (c-index = 0.71, 95% CI 0.67-0.74) and good calibration (R^2 value = 0.97), while BNP enhanced model performance (c-index = 0.74, 95% CI 0.70-0.78 and R^2 value = 0.91) (81). Especially for octogenarians, discharge NT-proBNP (over 1611 pg/mL) is highly correlated woith HF readmmisions and worse quality of life as assessed by the EQ-5D-3L questionnaire (82).

Although BNP levels may have an important predictive value in all time-points, i.e. on admission, during hospitalization, on discharge, or at follow-up of clinically stable chronic patients, a single BNP measurement may not be such a good predictor of the level of congestion compared to longitudinal assessments (83). In a subanalysis of the Italian Rapid Emergency Department (RED) study in 180 consecutive AHF patients with serial BNP measurements (on admission; at discharge; at 30, 90, and 180 days follow-up outpatient visit), a BNP higher than 400 pg/mL at

30 days after discharge, or the percentage variation of BNP from discharge to 30 days (Δ%BNP) showed a higher AUC and OR in predicting events [AUC=0.842, p<0.0001; OR 7.9 (3.3-19.0), p<0.001 for 30 days BNP and AUC=0.851, p<0.0001; OR 9.5 (4.065-22.572), p<0.0001 for Δ%BNP] compared with a BNP at discharge higher than 400 pg/mL [AUC=0.638, p<0.002; OR 2.4 (1.1-5.3), p=0.032] (84).

Similarly, in a study with 427 AHF patients, 3-month follow-up BNP and percent change in BNP were independently associated with all-cause mortality after adjustment for covariates, while admission value was not [discharge BNP integrated discrimination improvement (IDI) 0.072, p<0.0001, net reclassification improvement (NRI) 0.707, p<0.0001, and percent change in BNP (IDI of 0.113, p<0.0001 and NRI of 0.782, p<0.0001] (85).

A comparison of BNP and NT-proBNP in patients from PARADIGM-HF study (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure, 8399 patients in NYHA II-IV with LVEF ≤40% receiving recommended treatment) required to have BNP ≥150 pg/mL (or ≥100 pg/mL if hospitalized for HF within 12 months) or NT-proBNP ≥600 pg/mL (or ≥400 pg/mL if hospitalized for HF within 12 months) showed that each biomarker was similarly predictive of death (all-cause, CV, SCD and pump failure) and HF hospitalization (84), and the ratio of NT-proBNP to BNP appeared to be greater than generally appreciated, differed in the presence of AF, and increased substantially with age and renal impairment (86). Furthermore, apart from the prognostic accuracy of BNP in patients on ARNI treatment, there is evidence that the changes in BNP and NT-proBNP correlate with improvement in echocardiographic parameters of volume and function (87).

Concerning HF categories, BNP seems to be higher in HFrEF compared to HFpEF in similar congestion status as quantified by other markers (IVC diameter, atria size, and RV dysfunction - tricuspid annular plane systolic excursion) (88). In HFpEF high BNP levels may indicate high filling pressure since the ratio of early diastolic transmitral flow velocity and mitral annular velocity had strong positive

correlation with BNP levels, while in HFrEF it may indicate myocardial dysfunction as global longitudinal and circumferential strains were positively correlated (89).

Interestingly, in a study from Japan with AHF patients (n=748, 437 males) the prognostic value of plasma BNP values was different between sexes (90). Kaplan-Meier analysis revealed that in male high BNP values correlated significantly with worse prognosis than lower BNP values did, but this did not apply in female patients (90). In multivariate analysis, log transformed BNP at discharge predicted CV events in male but not in female HF patients (female, HR: 1.169; 95% CI: 0.981-1.399; p=0.08; male, HR: 1.289; 95% CI: 1.120-1.481; p=0.0004) (90).

NPs have also been correlated with echocardiographic features. In a study with HFrEF patients (n=117, median age 58 years, 80% males), doubling of NT-proBNP was associated with a decrease in LVEF by 1.83 (95% CI -2.63 to -1.03)%, p<0.0001; relative increase in mitral E/e' ratio by 12 (6-18)%, p<0.0001; relative increase in mitral E/A ratio by 16 (9-23)%, p<0.0001; decrease in TAPSE by 0.66 (-1.27 to -0.05) mm, p=0.03; rise in PASP by 2.74 (1.43-4.05) mmHg, p=0.001; and increase in LV and LA dimensions (p<0.05) (91). In a study with AHF patients (n=310), BNP levels linearly correlated with the degree of diastolic dysfunction and were significantly increased in patients with RV systolic dysfunction (TAPSE<18mm; p=0.006) and in patients with high propability of pylmonary hypertension (PASP≥40mmHg, p=0.001) (92). In a study with HFpEF patients (n=201), global longitudinal strain (GLS) was significantly associated with HF hospitalization and CV death both at rest [HR 0.91; 95% CI 0.84-0.98; p=0.016] and after exercise (HR 0.84; 95% CI 0.77-0.91; p<0.001) (93). The addition of exercise GLS and resting GLS improved the predictive power of BNP [net reclassification improvement (NRI) = 49%, p<0.001; NRI = 42%, p=0.004 respectively] (93). Similarly, the combination of BNP (with cut-off values of 200 pg/mL and 100 pg/mL) and global longitudinal strain (GLS with cut-off values of 7% and 14%) significantly stratified high-risk patients with known or suspected HF for SCD and MACEs respectively (94).

Exercise intolerance is a fundamental symptom in HF. A recent meta-amalysis of thirteen RCTs (n=2.997) showed that aerobic exercise significantly reduced NTpro-

BNP (weightened mean differences [WMD] =-741.69, 95% CI -993.10 to -490.27 [p<0.00001; I^2=63%]) and increased ventilatory efficiency, aerobic capacity, maximal workload, and LV function in patients with HF (95).

Surprisingly, in a study with HF patients (n=1.447 SR, N=1.151 and AF N=296) and a mean follow-up period of 1472 days, BNP was independently associated with HF events in SR (HR 8.08; 95 % CI 4.02-16.26; p < 0.0001), but less in AF patients (HR 1.97; 95 % CI 0.91-4.28; p = 0.087) (96). Furthermore, a significant causal relationship between increased NPs and AF occurrence has been ruled out (97).

Conserning the prognostic value of BNP in healthy subjects, a study with 30.487 participants (median age 63 years, 50% men, 17% black, 38% with HF) reported that in multivariate models including demographic and clinical characteristics, BNP and age were the strongest predictors of death in both patients with and those without HF; the risk for death according to BNP was similar between patients with and without HF, particularly in the acute care setting (98).

c) NP guided treatment

NPs have also been proposed to monitor and guide HF treatment (**Table 5**). The HOME-HF study demonstrated the feasibility of home BNP measurements and showed the potential value of the biomarker as an index of emerging clinical deterioration (99), and so did the HABIT trial in which 163 patients discharged for acutely decompensated HF measured daily BNP at home for 60days; the HR per unit increase of ln BNP was 1.84 (95% CI: 1.42 to 2.39) for the composite outcome: CV death, HF admission, diuretic treatment or changes in oral HF medications (100).

Several RCTs and meta-analyses have used NPs to approve or reject the use of medical treatments in HF. A meta-analysis of 5 RCTs (n=538, HFpEF patients) investigating the effect of beta-blockers on HF severity found no significant benefit in BNP levels, NYHA class or exercise capacity, unless there is CAD or AF (101). The TOPCAT (Treatment of Preserved Cardiac Function Heart Failure with an Aldosterone Antagonist Trial) trial had NP values as an inclusion criterion (BNP≥100 pg/mL or NT-

proBNP ≥360 pg/ml) and randomized patients with HFpEF (n=687) to spironolactone or placebo, and found that spironolactone was more effective in patients with lower NP values (102). In PARADIGM-HF trial, BNP levels increased after sacubitril/valsartan (ARNI) initiation (as is consists a substrate of neprilysin) while NT-proBNP decreased, however both biomarkers retained their prognostic accuracy (C-statistics of 63% to 67% for BNP and C-statistics of 64% to 70% for NT-proBNP) without difference, and increases values in both biomarkers after 8 to 10 weeks of ARNI were associated with worse outcomes (p = 0.003 and p = 0.005, respectively) (103).

However, there are RCTs that have failed to show benefit from the use of biomarkers (and specifically NPs) in HF monitoring and treatment. In GUIDE-IT trial, 1.100 chronic HFrEF patients with an HF-related event in the last year and NP values above a threshold (namely BNP > 400 pg/mL or NT-proBNP > 2000 pg/mL in the last month) were randomized to either a NT-proBNP-guided arm which aimed at NT-proBNP values less than 1000 pg/mL using guideline-directed medical therapy (GDMT), versus the usual care arm (104). In the biomarker guided arm, titration of neurohormonal antagonists was emphasized over titration of diuretics, unless there were signs of clinical congestion or very high NT-proBNP values (104). The trial found that NT-proBNP guided therapy was not more effective than usual care in improving the primary endpoint (CV death or HF hospitalization) for patients with chronic HFrEF (104). An interesting result of the GUIDE-IT population was that women had consistently lower NT-proBNP values, nevettheless, the change in NT-proBNP and clinical outcomes were similar to men (16). After adjustment, women achieving the NT-proBNP goal (below 1000 pg/mL) had an 82% reduction in death or HF hospitalization compared with a 59% reduction in men, which means that the early NT-proBNP goal of ≤1000 pg/mL had greater prognostic value in women (16).

In the TIME-HF trial (n=499, LVEF≤45%, various NYHA classes) the beneficial effect of NT-proBNP-guided therapy was seen only in subjects younger than 75 years (interaction terms with p=0.01 for all-cause hospitalization and p=0.03 for HF hospitalizations) (105).

A reason that the concept of biomarker-guided HF management did not show the positive results that it was expected to, might be the fact that HFrEF patients should receive optimal medical treatment regardless of biomarker results. However, contemporary data suggest that these patients are very often under-treated to GDMT goals (106). Therefore, biomarker-guided HF management could serve as a tool to optimize GDMT, by recognizing high risk patients when measured serially; rising values are associated with worse outcome whereas falling patterns could be reassuring (106). Another reason for failure might be the small sample size, ranging from 30 to 250 subjects per study arm (107). Although NP-guided HF therapy trials have had heterogeneous designs and mixed outcomes, a meta-analysis suggested a 20% to 30% mortality reduction associated with biomarker-guided HF management over usual HF care (108). Finally, a more recent meta-analysis of 18 trials (n=5.116) showed no significant difference in all-cause mortality (RR=0.91 [0.81, 1.03]; p=0.13), HF hospitalizations (RR=0.81 [0.65, 1.01]; p=0.06), and all cause hospitalizations (RR=0.93 [0.86, 1.01]; p=0.09) between the NP-guided versus guideline directed HF management (109), and the results were consistent both for NT-proBNP and BNP and irrespective of heart failure type and LVEF (109). However, sub-group analysis suggested that NP-guided treatment associated with less all-cause hospitalizations in patients aged less than 72 years (109).

An interesting policy that is rising is the combination of NPs with echocardiographic features (E/A ratio, E-wave deceleration time, pulmonary venous flow parameters like the systolic/diastolic wave velocity ratio and the difference in duration between pulmonary venous flow and mitral flow at atrial contraction, tissue Doppler imaging and left atrial volume index) to recognize congestion, identify high risk patients and guide HF treatment (110). A study with HFrEF patients (n=794, LVEF 34±9%, median age 69 years, 79% males, median follow-up 36 months) reported that at multivariate Cox regression analyses, major predictors of all-cause mortality were follow-up *E*/*e*' (HR: 1.05; *p*=0.0038) and BNP >125 pg/ml or decrease ≤30% (HR: 4.90; *p*=0.0054), while BNP > 125 pg/ml or decrease ≤30% and B-line numbers ≥15 were associated with the combined end point of death and HF hospitalization (111). The study concluded that optimal HF treatment, as guided by

the combination of serum biomarkers and echocardiogram was more effective in resolving pulmonary congestion while it was associated with better clinical outcomes (111). Furthermore, combined echocardiographic and NP guided management may reduce worsening of renal function in outpatients with chronic HFrEF (112).

d) Cost-effectiveness of NP usage in HF management

A very interesting Health Technology Assessment (HTA) analysis and systematic review in the UK, with data from 13 RCTs randomized 1536 HF patients to BNP-guided therapy and 1538 patients to symptom-guided therapy (113). According to this study, the efficacy of BNP-guided therapy in specialist HF clinics was found cost-effective only for patients younger than 75 years with HFrEF [HR 1.07 (95% CI 0.84 to 1.37), p=0.03] (113). Conversely, a systematic review of studies published between 2004 to 2017, regarding the cost-effectiveness of NP-guided treatment in HF patients reported that the use of NPs may reduce cost compared to the symptom-based clinical care and increase QALY, with the highest ratio of cost-effectiveness observed in the USA ($32,748) and the lowest in Canada ($6251) (114). Nevertheless, these studies came from high-income countries and the results in low- and middle-income countries might differ (114).

In conclusion, natriuretic peptides (mainly BNP and NT-proBNP) have been used extensively in HF management. They are proposed as the gold standard of biomarkers by the ESC and AHA HF guidelines, both for diagnosis and risk stratification and prognosis. Their role is maximized when used systematically and in serial time-points (i.e. on admission, during hospitalization, on discharge and during long term follow-up). They are used to guide HF treatment and monitor response to treatment. They are also used in clinical trials to confirm therapeutic results for pharmaceutical or other types of intervention (prosthetic valves, revascularization, transplantation, devices etc). They constitute a cost-effective intervention and their potential is even more promising with the use of point-of-care devices that are already available and can measure NPs more rapidly and easily, especially when used in addition to portable echocardiographic devices in primary care settings (115).

2. Troponin T and I

Troponin is a component of thin filaments (along with actin and tropomyosin) to which calcium binds to trigger the production of muscular force. Troponin has three subunits, TnC, TnI, and TnT, each playing a role in force regulation (116). There are variable isoforms of each subtype of troponin found within cardiac and skeletal muscle. Myofibrillar proteins such as Troponin T and I (TnT and TnI) are released in HF patients without an acute coronary event. Although these patients have underlying CAD, a detectable rise or fall of troponin may occur in the absence of significant epicardial coronary artery stenosis, indicating that other factors like the supply: demand mismatch are involved. The most recent high-sensitivity assays for troponins I and T (hs-TnI and hs-TnT) can detect serum concentrations up to 10 times lower than previous assays, even in asymptomatic subjects without previously known CV disease, rendering hs-Tn possible markers of subclinical myocardial damage in baseline conditions (117).

In a small study (n=30) with HFrEF patients on optimal medical therapy and without recognized inducible ischemia, that underwent maximal cardiopulmonary stress test, high sensitivity troponin T increased during exercise with a 4-hour peak ($p = 0.001$) and those patients with an hs-TnT increase greater than 20% were more symptomatic at rest ($p=0.039$), showed greater norepinephrine levels at peak exercise ($p=0.003$), and smaller cardiac output to NT-proBNP ratio increase ($p=0.034$), suggesting a pathophysiological link among transient hemodynamic overload, adrenergic activation, and myocardial cell damage (118). Moreover, higher troponin levels are associated with established HF risk factors like DM, LVH, CKD but not prior myocardial infarction or coronary calcium (119). What is of utmost importance is that elevated troponin values have a stronger relation with future risk of HF than ischemic events and serial measurements may improve risk classification (119). The latest ESC guidelines also suggest that baseline and serial troponin values should be assessed in patients presenting with AHF to assist in the diagnosis of various reasons and in treatment monitoring (4). Moreover, recent data support that hs-TnT and hs-TnI predict the risk of future CV events also in primary prevention and provide further information when added to current risk stratification models (117).

For instance, in a study with 19.501 Scottish general population both hs-TnI and hs-TnT were strongly associated with CVD risk in unadjusted models (120). After adjusting for classical risk factors, the HR for a 1-SD increase in log transformed troponin was 1.24 (95% CI, 1.17–1.32) and 1.11 (1.04–1.19) for cTnI and cTnT, respectively (120). Both hs-TnI and hs-TnT had strong associations with CVD death and HF while only hs-TnI was associated with MI and CAD (120). On the other hand hs-TnT was associated with non-CVD death, concluding that the associations of hs-TnI and hs-TnT with outcomes differ (120).

A meta-analysis of 16 studies (n=67.063) and 4.165 incident HF events showed that the participants with a baseline value of hs-cTn in the top third of the population had a pooled multivariate-adjusted HR for incident HF of 2.09 (95% [CI]: 1.76-2.48, p<0.001) compared to those in the bottom third) (121). There was no difference in HR for sex (men 2.29 [95% CI: 1.64-3.21] vs. 2.18 [95% CI: 1.68-2.81]), hs-cTnI and hs-cTnT (2.09 [95% CI: 1.53-2.85] vs. 2.11 [95% CI: 1.69-2.63]) and across other study-level characteristics (121). Further adjustment for BNP revealed a similar HR of 2.08 (95% CI: 1.64-2.65). When added to other conventional risk factors, hs-cTn improved the C index from 0.01 to 0.03. The data suggest a strong association of hs-cTn with HF prediction and identification of subjects at greater risk for developing HF (121).

The prognostic value of hs-TnT or hs-TnI for adverse events in men and women with stable HF and a preserved vs. reduced LVEF was studied in 853 HFrEF and 243 HFpEF patients (n=1096) enrolled in the Singapore Heart Failure Outcomes and Phenotypes (SHOP) study (122). Both hs-TnT and hs-TnI were more strongly associated with the composite endpoint (all-cause mortality or first HF rehospitalization) in HFpEF than in HFrEF (122). The hs-TnT assay yielded greatest additional prognostic value in HFpEF in comparison with hs-TnI and NT-proBNP while hs-TnI was more strongly associated with composite events in men with HFpEF [HR 3.33, 95% CI 1.82–6.09; $p < 0.001$ per standard deviation (SD) increase in log-transformed hs-TnI] than in women with HFpEF (HR 1.35, 95% CI 0.94–1.93; p=0.10 per SD increase in log-transformed hs-TnI) (122). Furthermore, lower values of hs-TnT and/or hs-TnI predicted higher frequency of reverse remodeling during follow-

up in patients with HfrEF, which in turn is accompanied by improved symptoms, better quality of life, and lower risk of hospitalization or death (123).

In a multi-center study with acutely decompensated HFpEF patients (n=34.233, 33.4% men; median age, 79 years) with measured troponin levels (78.6% with troponin I and 21.4% with troponin T), 22.6% (n = 7732) of patients had elevated troponin levels and that was associated with higher odds of in-hospital mortality ([OR] 2.19; 95% CI, 1.88-2.56), greater length of stay (more than 4 days OR, 1.38; 95% CI, 1.29-1.47), and lower likelihood of discharge to home (OR, 0.65; 95% CI, 0.61-0.71) independent of other clinical predictors and measured confounders (higher serum creatinine, black race, older age, and ischemic heart disease) (124). Elevated troponin I levels was also significantly associated with increased risk of 30-day mortality ([HR] 1.59; 95% CI, 1.42-1.80), 30-day all-cause readmission (HR, 1.12; 95% CI, 1.01-1.25), and 1-year mortality (HR, 1.35; 95% CI, 1.26-1.45) (124). However, in this very interesting study for the role of troponin in risk stratification in HF, what is also interesting is that from this large cohort of 96.769 patients only 35% had a troponin evaluation during hospitalization. Of those 34.233 patients with baseline troponin available, 14.819 patients had follow-up data available and only 35% of them had a troponin evaluation, which is a rather small percentage of all the data that could potentially be available (125). The study population finally is a minority of HFpEF patients rendering this fact an inherent study limitation.

Hs-Tn levels have been associated with echocardiographic features. In a study with HFpEF patients (n=70), hs-TnT concentrations correlated significantly with echocardiographically confirmed diastolic dysfunction (p=0.024) while hs-TnI increased only numerically (p=0.353) (126). In multivariable logistic regression models, hs-TnT concentrations >28 ng/L were associated with diastolic dysfunction grade III (OR=4.7, p=0.024), even after adjusting for NT-proBNP (126). Another study found that higher hs-cTnT levels were associated with greater LV mass index (p<0.001) and with worse diastolic function, including lower tissue Doppler imaging e' (6.00 [0.07] vs 5.54 [0.06]; p<0.001), higher E/e' ratio (11.4 [0.2] vs 12.9 [0.1]; p<0.001), and greater left atrial volume index (23.4 [0.4] vs 26.4 [0.3]; p<0.001), independent of LV mass index; furthermore, hs-cTnT value was not associated with

LV systolic function (127). In patients with severe aortic stenosis undergoing transcatheter aortic valve replacement (n=105), troponin and hs-TnI correlated significantly with LV mass index, and could predict overall mortality in a medial follow-up of 375 days (Log-rank p=0.03 for both) (128).

A Spanish multicenter study (EAHFE-TROPICA3 study) analyzed the characteristics and outcome of AHF patients presenting in the emergency department, in whom cTn was not determined based on clinical criteria that classified them as low-risk for in-hospital or 30-day mortality (129). Data from 8850 patients with AHF were analyzed and cTn was not determined in 47.6% of them. These patients had a lower prevalence of IHD, used loop diuretics more frequently but were sub-optimally treated with other medications established for HF management (angiotensin-converting enzyme inhibitors, aldosterone receptor antagonists, and beta-blockers) and had a greater rate of edema in the acute episode. Compared with the group of patients that cTn was estimated, this group had the same in-hospital mortality (adjusted odds ratio [OR] 1.21, 95% confidence interval [CI] 0.98—1.50), mortality at 30 days (adjusted OR 1.07, 95% CI 0.90—1.28) and reconsultation at 30 days (adjusted OR 0.90, 95% CI 0.80—1.02), thus was not related to a better prognosis (129). This study suggests that cTn should be measured in all AHF patients, to improve the stratification prognosis according to the presence or absence of high cTn concentrations (129).

Across literature hs-TnI is an independent predictor of HF outcomes (128). It has been studied in HFrEF, HFmrEF and HFpEF patients (131-133), and it has been correlated with adverse LV remodeling in chronic HF (134). Elevated hs-TnI one-month post discharge of patients with acutely decompensated HF was independently predictive of increased all-cause mortality [HR 1.59, 95% (CI) 1.18-2.13] and CVD or HF hospitalization (HR 1.28, 95% CI 1.03-1.58) at 12 months (135), while in the APIC study that observed 8.121 apparently healthy subjects for a longer period (median follow up of 15 years), an elevated hs-TnI >3.8 pg/ml predicted HF hospitalization (HR, 4.20; 95% CI, 3.28-5.37), and all-cause mortality (HR, 1.83; 95% CI, 1.56-2.14) (136).

In patients with HFpEF (n = 38) that underwent cardiac catheterization, echocardiography, and expired gas analysis at rest and during exercise, hs-TnT were more than 2-fold higher at rest and during exercise ($p< 0.0001$) and significantly correlated to LV filling pressures ($r = 0.52$; $p<0.0001$) and diastolic function ($r = -0.43$; $p=0.002$), reduced cardiac output ($r = -0.44$; $p= 0.002$), and worse aerobic capacity (137). In patients with stable HFrEF, a 12 week exercise program was associated with reduced hs-TnT (138). In a recent meta-analysis in HFrEF patients (n=9289) hs-TnT had a median value of 16 ng/L (8-20; n = 9289) and was independent predictor of all-cause and cardiovascular mortality and cardiovascular hospitalization, with stronger predicting ability than NT-proBNP (139). In AHF admission hs-TnT values ≥43 ng/L hold independent prognostic significance for post-discharge outcomes (140). In the British Regional Heart Study men without baseline HF (n=3852), aged 60-79 years were followed for a median of 12.6 years. A 1-SD increase in log-transformed hsTnT was associated with a higher risk of incident HF after adjusting for classic risk factors (HR 1.58, 95% CI 1.42-1.77) and after additional adjustment for NT-proBNP (HR 1.34, 95% CI 1.19-1.52) (141). HsTnT values <5ng/L had a sensitivity of 99.7% (95% CI 98.1%-99.9%) and a specificity of 3.4% (95% CI 2.8%-4.0%) (141).

The HFrEF patients enrolled in the Alberta Heart Failure Etiology and Analysis Team (HEART) Study that improved the LVEF by more than 5% (316 out of 621) in one year had evidence of further late improvement in LVEF and unchanged hs-TnT levels, while those patients (305 out of 621) that had persistent HFrEF without LVEF improvement increased hs-TnT levels over time (142). These data help to inform mechanisms relating to late LV remodeling and the prognostic role that this biomarker may have (142).

These data confirm the strong prognostic role that troponin has in all HF spectrums, in acute and chronic clinical settings that render it probably the second most valuable biomarker after NPs.

3. Galectin-3 (Gal-3)

Galectins are β-galactoside-binding lectins; 15 types have been currently identified in mammals. Among them, galectin-3 (gal-3) is a 35-kDa protein that is coded by the gene LGALS3 which is located on chromosome 14, and it is the only chimera-type galectin. It is widely expressed in human tissues including all types of immune cell (macrophages, monocytes, eosinophils, mast cells, natural killer cells, activated T and B cells). Gal-3 is predominantly located in the cytoplasm and it is involved in several biological activities including cell growth, apoptosis, pre-mRNA splicing. Gal-3 as a biomarker of fibrosis and inflammation has also been involved in the pathogenesis of cardiovascular remodeling and progression of HF (143). Baseline serum gal-3 has been shown to have prognostic value in regards to mortality (144, 145) and re-hospitalization (146), while changes in gal-3 levels have prognostic value in regards to morbidity and mortality (147) and cardiovascular events (148).

Increased levels of gal-3 were also associated with clinical severity as expressed by NYHA class and HF phenotype (149). In HFrEF patients (n=67) on optimal medical therapy, higher gal-3 values correlated significantly with worse RV systolic dysfunction (s', TAPSE), higher pulmonary artery systolic pressure, worse tricuspid regurgitation, lower RV-to-pulmonary circulation coupling index, and lower parameters in cardiopulmonary exercise testing (150). Multivariate regression analysis revealed that TAPSE (β coefficient: - 0.605; $p<0.001$) and heart rate at peak exercise (β coefficient: - 0.98; $p=0.009$) were independently related to gal-3 concentration, indicating concomitant RV dysfunction and exercise intolerance (148). However, another study with HFrEF patients (n=132) found no association between gal-3 levels and echocardiographic features like LVEF, LV mass index, LV internal diameter in diastole (LVIDd), early diastolic mitral flow to annular velocity (E/E') or LV global longitudinal strain ($p>0.05$ in all cases) (151). In another study with 128 elderly patients with HFrEF, circulating levels of gal-3 were significantly and independently associated with frailty (OR = 5.64; 95% CI = 1.97-16.22) (152). Finally, there are no significant differences in gal-3 levels among HFpEF and HFrEF patients (153).

In pediatric populations with congenital heart disease (n=75), gal-3 was raised compared to healthy adult controls, and it showed significant increase when HFrEF was present (p=≤0.001) compared to congenitl heart disease without HF (154). Gal-3 was positively correlated with Ross classification (r = 0.68, p = 0.018) and negatively correlated to LVEF (r= -0.61, p ≤ 0.001). Importantly, gal-3 had better diagnostic value than Ross HF classification [96.7% sensitivity, 90% specificity, 91% positive predictive value, 93.2% negative predictive value, with area under the curve (AUC = 0.96) and 93% accuracy] (154). Contrary to adult HF populations, there was no significant correlation between serum gal-3 level and HF mortality (p = 0.08) (154).

Two recent large meta-analyses show that gal-3 is associated with higher all-cause mortality (155, 156). The first one showed significant association in both acute and chronic HF patients, [HR 2.17 (95% CI:1.27-3.08 and) 1.13 (95% CI,:1.07-1.21)] respectively (155), while in the second one gal-3 was associated with higher all-cause mortality [(adjusted risk ratio [RR], 1.58; p<0.001)], mortality/HF re-hospitalization (RR, 1.68; p<0.001), and cardiovascular mortality [(RR, 1.29;p=0.04)], but not HF re-hospitalization [(RR, 1.24; p = 0.25)] in AHF patients (156).

In patients with advanced HF and need for extracorporeal life support (ECLS) Gal-3 (along with sST2) have important prognostic efficiency while other biomarkers like BNP, NT-proBNP, TnI, MR-proANP, proADM, and copeptin have failed to show significance (157).

The ACC/AHA guidelines recommended the use of Gal-3 for the assessment of cardiac fibrosis in heart failure; however, thus far, the ESC has not recommended the clinical use of Gal-3 (60) although there are studies published in ESC journals pointing towards that direction, like a study with AHF patients (n=80, median age 52.6 years, males 47.5%) in which gal-3 at baseline and its changes in serial measurements were significantly associated with changes in symptoms, echocardiographic remodelling, CV death or HF hospitalization during a six-month follow-up (158).

In conclusion, Gal-3 may not constitute an organ-specific marker; however it is a specific marker of pathogenesis, such as macrophage-related disease or fibrosis

(159). Thus, the cardiac-infiltrating macrophages expressing gal-3 in the early stages are potential therapeutic targets for cardiac fibrosis and remodeling in HF (159) rendering gal-3 an important diagnostic and prognostic biomarker.

4. Soluble Interleukin 1 receptor-like 1 (ST2)

ST2 is a member of the interleukin (IL)-1 receptor family. Several studies have illustrated its prognostic role both for AHF and CHF (160), while the American College of Cardiology Foundation and American Heart Association recommend measurement of soluble (s)ST2 for risk stratification in patients with AHF (class IIb, Level of Evidence:A) and in patients with chronic HF (class IIb, Levelof Evidence: B) (161). ST2 values are not significantly affected by body mass index (BMI) or by renal impairment, a major limitation for BNP or NT-proBNP (162, 163). The potential diagnostic role of sST2 in AHF was investigated in the PRIDE (Pro-Brain Natriuretic Peptide Investigation of Dyspnea in the Emergency Department) study (164). The optimal ST2 cutpoint was 0.20ng/mL, producing an area under the curve (AUC) of 0.74 for the diagnosis of AHF (95% confidence interval [CI] of 0.70–0.78; $p<0.001$) (164). In the same study ST2 was illustrated to have prognostic value as well. sST2 values were higher in patients who died within one year compared to survivors ($p<0.001$), while greater values predicted higher risk (164). A recent meta-analysis confirms the prognostic value of ST2 in CHF (165). This analysis consists of 7 trials with 6372 CHF patients and sST2 had an HR of 1.75 (95% CI 1.37–2.22) for all-cause death and 1.79 (95% CI:1.22–2.63) for CV death (both P<.001). In a study with CHF patients (n=4268), sST2 level was also independently associated with all-cause mortality, cardiovascular death and HF hospitalization in models including several clinical prognostic characteristics (age, sex, BMI, ischemic etiology, LVEF, NYHA class, e-GFR, therapy with ACE inhibitor and/or ARB, beta-blocker, MRA), as well as in the same model combined with either NT-proBNP or hs-TnT alone, adding extra prognostic value to these biomarkers (166). In CHF sST2 serial measurements independently added prognostic information to baseline concentrations (OR: 3.64;

95% CI: 1.37 to 9.67; p=0.009) and predicted reverse myocardial remodeling (OR: 1.22; 95% CI: 1.04 to 1.43; p=0.01) compared to GDF-15 and hsTnT (167).

ST2 was used to reflect different pathophysiological pathways between HFpEF and HFrEF patients (n=570) and to investigate if it's prognostic value (for 18-month overall and HF hospitalization-free survival) would be different (5). ST2 was higher in HFpEF patients [37.6 (28.5–54.7) vs. 35.7 (25.6–52.2), p = 0.02]. Moreover, ST2 improved the area under the curve from 0.86 (0.82–0.89) to 0.91 (0.87–0.94; *P* < 0.001) for discriminating HFpEF from HFrEF. However, there were no significant differences regarding the prognostic value of ST2 between HFpEF and HFrEF (5).

ST2 has also been investigated as a sudden cardiac death (SCD) and ventricular arrhythmia predictor in HFrEF (168, 169). ST2 was higher among patients who died (0.23 ng/mL [IQR 0.16–0.43 ng/mL] vs 0.12 ng/mL [IQR 0.06–0.23 ng/mL], P 5 .001) and were predictive of SCD (10.1 ng/mL, odds ratio 1.39, 95%CI 1.09–1.78, P=0.006) (168). ST2 was found to be predictive of ventricular arrhythmia in a cohort of 684 patients enrolled for the MADIT-CRT Trial (HR [95% CI] =1.10 [1.01–1.20]; p= 0.029) and death or ventricular arrhythmia (HR [95%CI] =1.11 [1.04–1.20]; P=0 .004) (169).

Another valuable role for sST2 was its response to medications, rendering it usefyl for HF monitoring. Patients with an higher baseline sST2 concentration who achieved higher beta blocker doses (metoprolol succinate equivalent >50 mg/d) had substantially lower risk for CV events compared with patients that tpolerated lower beta blocker dose, while patients with lower initial sST2 values had smaller relative and absolute benefit from beta blockers, even in high doses (170). Furthermore, patients with lower sST2 levels had greater reduction in the risk of death or HF with CRT-D than subjects with higher sST2 (p=0.006) (169). The combination of elevated sST2 and gal-3 in stable CHF patients (n=101) treated with MRAs is associated with poorer 1-year adverse prognosis compared to either alone [HR = 4.4 (95% CI 1.9-8.9) versus 7.3 and 6.6 respectively] (171). This prognostic capacity is significant and independent of BNP levels and MRA therapy (171). Another study that combined sST2, gal-3, GDF-15 and BNP found that the three biomarkers (exept BNP) can identify patients with HFpEF compared to controls but cannot differentiate HFpEF

from HFrEF and an index is needed that incorporates GDF-15, Gal-3, or sST2 with BNP (153).

In patients with advanced HF and need for extracorporeal life support (ECLS) sST2 (along with Gal-3) have important prognostic efficiency while other biomarkers like BNP, NT-proBNP, TnI, MR-proANP, proADM, and copeptin have failed to show significance (157).

As reported by a study with CHF (n=5301), a major advantage of ST2 is that unlike NPs and hsTnT, it is not affected by age (172). In this study, the best NT-proBNP and hs-TnT cut-offs for 1-year and 5-year all-cause mortality and cardiovascular death, and 1-year HF hospitalization increased with age, while the best sST2 cut-offs did not (172). ST2 is also not affected by renal function or BMI (162) and taken all the above into account, ST2 is robust biomarker with prognostic role in HF, also supported by the American guidelines (161). Future studies are expected to strengthen its role to therapeutic decision-making in both acute and chronic HF.

5. Fibroblast Growth Factor 23 (FGF-23)

Fibroblast growth factor 23 (FGF-23) is primarily secreted by osteocytes to maintain phosphate and mineral homeostasis. Experimental studies demonstrate that increased hormone levels associate with pathologic cardiac remodeling like LVH and myocardial fibrosis, independent of preserved or reduced renal function (173). FGF-23 was measured in 379 HFpEF participants from TOPCAT trial (174). Along with 48 other plasma biomarkers, using a multiplex assay, they assessed the relationship between biomarkers and the risk of all-cause death or heart failure-related hospital admission. Six clusters were found, with biomarkers of fibrosis/tissue remodeling (MMP-2, 3 and 9, Galectin-3), inflammation liver fibrosis, renal injury (NGAL, cystatin C), neurohormonal regulators (FGF-21, GDF-15, FGF-23), myocardial injury/stretch (troponin T and hFABP, NT-proBNP), and inflammatory mediators (TNF-a, ST2). FGF-23 demonstrated the strongest association to adverse outcomes compared to all above mentioned biomarkers, which was also independent of the MAGGIC risk score, rendering it a potentially useful biomarker for HF prognosis.

In patients with non-ischaemic HF and preserved renal function (n=208), FGF-23 was independently related with the combined endpoint of death or heart transplantation (HR 1.452 [1.029-2.048]; p = 0·034) (173). A dose-response relationship was found between median FGF-23 values and NYHA class (I: 11·9 RU/mL, II: 15·8 RU/mL, III/IV: 38·8 RU/mL; P < 0·001). FGF-23 correlated with NT-proBNP (r = 0.307, p< 0.001), central venous pressure, mean pulmonary arterial pressure, pulmonary capillary wedge pressure and inversely correlated with cardiac output (175). In HFpEF, apart from NYHA class FGF-23 was found to correlate with other markers of exercise capacity such six minute walk test (6MWT) distance, (r=-0.345), and markers of diastolic function (echocardiographic E/e' (r= 0.298); p<0.05) (176) while peak oxygen consumption was also adversely correlated with FGF-23 (177). The discharge FGF-23 value also has been correlated to one year mortality and HF hospitalization outcome in patients admitted AHF (178).

FGF-23 has also been studied in patients with arterial hypertension (n=2858), a major risk factor for HFpEF development. Higher baseline FGF-23 was associated with a 63% greater hazard of incident HF (HR: 1.63, 95% confidence interval: [1.13-2.36] over a 14-year median follow-up, which persisted even after exclusion of participants with chronic kidney disease (HR: 1.94 [1.10-3.43]) rendering it a useful biomarker for HF risk in hypertensive populations (179). In pediatric patients with DCM, higher FGF2-3 levels were associated with a greater left ventricular end-diastolic diameter (r = 0.63, P = 0.04) and disease severity (180).

Apart from plasma FGF-23 has also been found elevated in bone marrow plasma in patients with CHF (n=203) compared to controls and higher than the respective plasma values from HF patients (p<0.001). FGF-23 bone marrow values correlated significantly with LVEF (r = -0.37), NT-proBNP (r = 0.6) NYHA class (r = 0.28), and eGFR (r = -0.43), (p<0.001). Bone marrow FGF-23 was also independently associated with all-cause mortality (181). On the contrary, myocardial FGF23 gene expression was measured and found similar in HF patients and controls, and immunohistochemistry showed similar cellular distribution in both groups, suggesting that the myocardium does not contribute to the elevated circulating FGF23 in HF (182).

On the contrary, in a multicentre trial with symptomatic HF patients (n=622) FGF-23 significantly predicted all HF-related outcomes at baseline, 3, 6 and 12 months in unadjusted analyses, but after multivariable adjustments, the association lost statistical significance apart from month 3 (183). However patients with HFpEF and HFmrEF had higher levels of FGF-23 than patients with HFrEF, and perhaps there is an heterogeneous role of FGF-23 in different LVEF groups which deserves further investigation (183).

6. Copeptin

Vasopressin is released from the posterior pituitary lobe in case of increased plasma osmolality or reduced systemic blood pressure (184). Although the vasopressin system is up-regulated in HF, it is difficult to measure vasopressin because of its small size and short half-time (24 minutes). An acceptable method of quantifying vasopressin levels is measuring the plasma concentration of the C-terminal segment of the precursor peptide, which is called copeptin and has a longer half-time (185). A recent meta-analysis of ten prospective cohort studies (n=4473) with HF patients showed that elevated copeptin was associated with an increased risk of all-cause mortality in HF patients (RR was 2.64 (95% CI, 2.09-3.32)), with a pooled sensitivity and specificity 0.57 (95% CI, 0.50-0.63) and 0.74 (95% CI, 0.69-0.79) respectively (184). The positive likelihood ratio was 2.2 (95% CI, 1.90-2.60) and the negative likelihood ratio was 0.58 (95% CI, 0.52-0.66). Furthermore, the predictive value of copeptin was found comparable with NT-proBNP for all-cause mortality in HF patients (186).

A second meta-analysis of 13 studies showed similar results, though with a borderline positive association of plasma copeptin with HF risk (HR, 1.60; 95% CI, 0.90-2.85) and a significant association between plasma copeptin and mortality of HF (HR, 1.76; 95% CI, 1.33-2.33) (187). Furthermore, an increment in copeptin of 1 pmol/l was associated with a 3% increase in all-cause mortality (HR, 1.03; 95% CI, 1.01-1.05), concluding that elevated plasma copeptin is associated with an increased risk of HF and all-cause mortality in HF patients (187). A third meta-analysis with 5.989 participants from 17 prospective studies also revealed a significant association

between copeptin levels and risk of all-cause mortality in HF patients (categorical copeptin: HR =1.69, 95% CI =1.42-2.01; per unit copeptin: HR =1.03, 95% CI =1.00-1.07; log unit copeptin: HR =3.26, 95% CI =0.95-11.25) (188).

One study compared the copeptin values among different groups of HF. The copeptin levels were higher in HFrEF group (n=131) than in the HFpEF group (n=127) and both were higher than controls (n=119). The copeptin levels were positively correlated with the NT-proBNP levels in HFrEF and they increased as the NYHA class increased; however, there was no correlation between the copeptin and NT-proBNP values in HFpEF patients and the copeptin levels did not change markedly as the NYHA class increased (189).

Elevated copeptin was found to correlate significantly with later development of HF in an older middle-aged population (n=5297) with a mean follow-up of 11.1 years, independently of established risk factors and after adjustment for kidney function (190). Copeptin has been found to have a prognostic role in the acute setting as well (191). In patients with AHF (n=1697), copeptin was highly prognostic for 90-day adverse events (combined end points of mortality, HF readmissions, and emergency department visits) adding prognostic value to clinical predictors, serum sodium, and NPs (192, 193). Similarly, when compared with other biomarkers of HF, in patients with acute and decompensated CHF (n=164) copeptin at admission with re-measurement at 72h was found to be the best predictor of 90 day mortality and rehospitalization (χ^2 = 16.63, C-index = 0.724, $p < 0.001$) (194). NT-proBNP, mid-regional pro-atrial natriuretic peptide (MR-proANP), mid-regional pro-adrenomedullin (MR-proADM), and C-terminal pro-endothelin-1 (CT-proET1) were also measured on admission, after 24, 48, and 72h, and every 72h thereafter, at discharge and then in follow-ups. Copeptin at admission was the best predictor of 90 day mortality or rehospitalization, followed by NT-proBNP (χ^2 = 10.53, C-index = 0.646, p=0.001), while the other biomarkers also had very good results (194). A second measurement of copeptin at 72h and NT-proBNP at 48h further increased prognostic value (χ^2 = 23.48, C-index = 0.718, p=0.00001; χ^2 = 14.23, C-index = 0.650, p=0.00081) respectively (194). A meta-analysis of 45 studies concerning the

diagnostic value of biomarkers such as copeptin, galectin-3, hs-cTnT, MR-proANP, MR-proADM, and ST2 found that copeptin had the lowest negative predictive value (0.70 [95% CI: 0.66-0.74]) (195).

Copeptin has also a prognostic role in more specific causes of HF. Women with peripartum cardiomyopathy (n=28, median follow up 91 months) had higher levels of copeptin, which was inversely correlated with maximal exercise capacity and cardiac functional parameters assessed with cardiac magnetic resonance imaging (196). Furthermore, copeptin was progressively lower in 13 patients one year post left ventricular assist device (LVAD) and in 22 patients one year after heart transplantation (HTx) compared to baseline, suggesting that improvement in cardiac output with LVAD and HTx may induce progressively reduced activation of vasopressin (197). In 65 advanced HF patients referred for right heart catheterization prior to HTx, increased plasma copeptin levels were associated with hemodynamic parameters in particular-reduced cardiac index ($r = 0.65$ and $p = 0.04$), rendering copeptin a useful biomarker for resting hemodynamics in HF (198).

Taken all this data into account, copeptin is a very useful biomarker for short and long term prognosis in HF of various causes that may add prognostic value to clinical predictors and other biomarkers such as NPs.

7. Growth Differentiation Factor-15 (GDF-15)

Growth Differentiation Factor-15 (GDF-15) is secreted as a pro-peptide that is cleaved in the endoplasmic reticulum to an active peptide that enters the circulation. Increased expression of GDF-15 leads to reduced expression of phosphorylated RelA p65 pro-inflammatory and pro-apoptotic genes, and increased Foxo3 phosphorylation, which limits myocardial tissue damage and apoptosis (199). Furthermore, GDF-15 enhances hypertrophic cardiomyocyte cell growth (200) and inhibits growth hormone signaling (201).

Most of the studies of the association of GDF-15 with HF or its short and long term prognostic ability in HF are taking into consideration other factors such as NPs as well, and they include mostly patients with AHF or HFrEF. One study reported that

increased levels of GDF-15 at days 2 and 14 of hospitalized patients with AHF have been associated with a greater risk of 60-day HF/renal failure rehospitalizations/cardiovascular death and cardiovascular death at 180 days (202). A second study with AHF patients reported that higher GDF-15 values are associated with worse prognosis independently of BNP, and when both GDF-15 and BNP were elevated at discharge, the 2-year mortality risk increased over four-fold (203). In patients with STEMI increased GDF-15 values are strong predictors of death and HF in six months (204).

There are also studies with CHF but comparatively fewer (205-207). Interestingly in one study with 209 patients with CHF mostly NYHA III class, GDF-15, hs-CRP, Gal-3, hs-TnT, and NT-proBNP were measured and related to all-cause long-term mortality (208). Each marker was predictive for mortality in univariate analysis. In multivariate analysis, elevated concentrations of GDF-15 (hazard ratio [HR] 1.41, confidence interval [CI] 1.1 to 178, p = 0.005), hs-CRP (HR 1.38, CI 1.15 to 1.67, p = 0.001), and hs-TnT (HR 1.27, CI 1.06 to 1.53, p = 0.008) were independently related to mortality. All novel markers had an incremental value to NT-proBNP, using the integrated discrimination improvement and were found as independent prognostic markers, but GDF-15 was the most predictive marker, even stronger than NT-proBNP (208). A recent meta-analysis of CHF patients that compared the prognostic value of GDF-15, hs-CRP and sST2 found that sST2 was predictive of short-term mortality (P = 0.0225), and GDF-15 was more sustained over time and was predictive of middle term mortality (P = 0.0375) (209).

There is comparatively little data that focuses on HFpEF. Circulating GDF-15 levels are higher in patients with HFpEF compared to controls (153). A study with a subgroup of patients from the DIAST-CHF observational trial compared HFpEF (n=142) with controls (n=188) and found considerably higher values in patients with HFpEF at 1.66 ng/mL (interquartile range [IQR]:1.26–2.34 ng/mL) vs 0.9 ng/mL (IQR: 0.70–1.09 ng/ mL) in controls (p < 0.0005) (210). GDF-15 levels were higher in patients with HFpEF (n=99) referred for elective coronary angiography compared to controls (n=20) and were higher in those with moderate to severe (n=38) compared to mild diastolic dysfunction (n=61) (211), although there was overlap of values of those with normal diastolic function compared to those with mild diastolic

dysfunction. In a study from six public institutions serving the majority of the Southeast Asian population of Singapore, comparing GDF-15 in patients with HFpEF (n=50) and controls (n=50), the combination of GDF-15 and NT-proBNP was not statistically different from either GDF-15 alone or NT-proBNP alone (212).

Regarding the ability of GDF-15 to differentiate HFpEF from HFrEF, most of the studies conclude that GDF-15 plasma values in HFpEF are comparable to HFrEF, but not as high as the in HFrEF (5, 213). Indicatively in one study a small but not significant difference of median GDF-15 in individuals with HFpEF (n = 108) was found compared to those with HFrEF (n = 38) at 1.29 ng/mL (IQR: 0.99–1.87 ng/mL) compared to 1.51 ng/mL (IQR: 1.09–2.13 ng/mL), respectively (213). A study that compared directly the clinical correlates and prognostic value of GDF15 between HFpEF vs. HFrEF found similar medium baseline values [2862ng/L (1812, 4176)] vs. [2517ng/L (1555, 4030)] respectively (P = 0.184), whereas NT-proBNP was significantly lower in HFpEF than HFrEF (1119 ng/L vs. 2335 ng/L, P < 0.001) (214). During a 23 month follow-up there were 379 events (72 HFpEF vs. 307 HFrEF). GDF-15 remained a significant independent predictor for composite outcome even after adjusting for hsTnT and NT-proBNP (adjusted HR 1.76, 95% CI 1.39–2.21; P < 0.001), regardless of LVEF (P= 0.275) and provided incremental prognostic value when added to these biomarkers, with a net reclassification improvement of 0.183 (P = 0.004), suggesting that beyond haemodynamic compromise (NT-proBNP), inflammatory injury (GDF15) may play an important role in HF (214).

8. Endothelin-1 (ET-1)

Enthothelin-1 (ET-1) is a 21-amino acid protein that has prolonged vasoconstrictor properties (215). It is produced by cardiac myocytes and vascular endothelial cells, and increases the contractility of cardiac muscles and vascular smooth muscles. It may also cause myocardial hypertrophy and cellular injury (216). Endothelin-1 (ET-1) concentrations are elevated in patients with congestive heart failure; this happens because of increased production (0.21±0.03 in CHF vs. 0.06±0.02 $ng \cdot kg^{-1} \cdot min^{-1}$ in controls; $p<0.05$) rather than decreased clearance (0.119±0.018 in CHF vs. 0.047±0.013 $l \cdot kg^{-1} \cdot min^{-1}$ in controls, p=0.05) (217).

ET-1 and has been found to have prognostic value both in AHF and CHF (218-220). A meta-analysis of thirty-two studies with 18,497 patients showed that circulating ET-1 was positively correlated with high risk of adverse outcomes, with a pooled RR (95% CI) of 2.22 (1.82-2.71, p<0.001). In the subgroup of death as primary outcome, the pooled RR (95% CI) was 2.13 (1.68-2.70, p<0.001). No significant publication bias was observed in this study (221).

One study reported that plasma ET-1 at baseline and 48-72 h was found to be independently associated with 180-day mortality in a multivariable analysis [HR 1.6, 95% CI 1.3-2.0, p<0.001 and HR 1.5, 95% CI 1.2-1.9, p=0.001, respectively] in AHF patients (n=872) (222). ET-1 at 48-72 h was also independently associated with death or worsening HF prior to discharge [OR 1.6, 95% CI 1.03-2.4, p=0.03], with these associations remaining significant after including NT-proBNP in the multivariable analysis (222). Although this study did not take the potential impact of increased BMI (overweight and obesity) and obstructive sleep apnea, both known to be associated with increased ET-1-mediated vasoconstriction (223), its results are in concordance with other studies, that have found even better prediction of survival compared to NPs (224). ET-1 has been positively correlated to NT-proBNP (p<0.0005) (225). C-terminal proendothelin-1 (CT-proET-1) is the stable circulating precursor protein of ET-1 and it has also been increased in CHF patients and correlated with NYHA class (226).

What is of utmost importance is that the prognostic value of ET-1 applies both for HFrEF (OR=2.9, CI=1.1-7.7 for mortality and HF hospitalizations) (227) and HFpEF (OR 1.4, 95% CI 1.04-2, p=0.03 for 1 year HF hospitalization) (228). Furthermore, ET-1 may serve as a potential prognostic marker for developing PH in HFpEF (229) and is also significantly correlated with lower exercise oxygen consumption both at baseline and longitudinally over 24 weeks in patients with HFpEF (n=210) (230).

Concerning arrhythmias and cardiac resynchronization treatment (CRT), in 367 patients receiving CRT, ET-1 value greater than 0.56 pmol/L was an independent predictor for primary endpoint (all cause mortality and heart transplantation) [HR:

2.005, 95% CI 1.045-6.2621, p=0.040] and HF hospitalization (HR = 2.126, 95% CI 1.182-3.827, p=0.012) (231). However, in HFrEF patients with ischaemic or dilated cardiomyopathy (n=160 plasma levels of ET-1 were not associated with arrhythmic death or resuscitated cardiac arrest (HR = 1.07 [0.98-1.17], p=0.113) but with a higher risk for overall mortality (HR = 1.64 [1.29-2.08], p < 0.001) (232).

In conclusion, ET-1 is a reliable prognostic biomarker both for short and longer outcomes in AHF but also for CHF as well.

9. C-reactive protein (CRP)

C-reactive protein (CRP) is an acute phase protein and one of the commonest non-specific inflammatory response markers. Secreted and synthesized by the liver, this biomarker is significantly associated with disease severity and cardiomyocyte necrosis in several CV diseases (233). Specifically for HF, elevated CRP levels can also be stimulated by circulating macrophages, monocytes, and leukocytes (234). The most recent high-sensitivity assays for CRP (hs-CRP) can detect even lower concentrations in asymptomatic subjects, and may therefore strengthen the prognostic role of hs-CRP in HF. In the British Regional Heart Study, subjects without prevalent MI or HF (n=3569) were followed for mean 16.3 years; those in the highest baseline CRP had an elevated risk of HF after age and BMI adjustment (HR = 1.42 [1.01-1.98]), which markedly attenuated after NT-proBNP adjustment (HR = 1.15 [0.81-1.63]) (235). Furthermore, CRP was significantly and positively associated with NT-proBNP (235). In a study in Sweden with men aged 50 years in 1993, elevated hs-CRP levels were predictive for HF over a 21-year follow-up (236).

In chronic HF raised hs-CRP value was significantly associated with worse clinical status and prognosis (237). The median [interquartile range (IQR)] plasma hs-CRP for HF patients (n = 3756) was 3.9 (1.6–8.5) mg/L and 2.7 (1.3–5.1) mg/L for controls (n = 667; P < 0.001). Patients with higher hs-CRP were older and more congested (237). Higher plasma hs-CRP values were associated with greater mortality, independent of age, symptom severity, creatinine, and NT-proBNP (237). Comparing a hs-CRP ≥10 mg/L to <2 mg/L, the hazard ratio for all-cause mortality

was 2.49 (95% confidence interval 2.19-2.84; P < 0.001), for CV mortality was 2.26 (1.91-2.68; P < 0.001), and for non-CV mortality was 2.96 (2.40-3.65; p<0.001)(237). In CHF temporal patterns representing evolution of level and rate of change in level of CRP level were independently associated with adverse prognosis expressed as HF hospitalization, CVD, cardiac transplantation, and LV assist device implantation [multivariable-adjusted HR per doubling of biomarker: 1.65 (1.30-2.08)] (238). Instantaneous change in biomarker level was also independently associated with the primary outcome (238).

In AHF (n=527 hospitalized patients) the hazard ratio (HR) for long-term mortality significantly increased with admission CRP levels in a dose-dependent manner during a median follow-up period of 24 months (p for trend = 0.034) in the multivariable analysis (239). Likewise markedly elevated CRP admission levels in patients with AHF may be associated with higher short-term cardiac and non-cardiac mortalities after a 4 month follow-up (240). In hospitalized patients with AHF (n=257), an increased CRP to prealbumin ratio (>1.60) had a significantly greater risk of all-cause, cardiac, and non-cardiac death (log-rank test, all p<0.001) than patients with lower ratio (241). Although the prognostic role of CRP at admission may be confounded by an acute-phase response, CRP levels at discharge have also a prognostic value in AHF (n=4269) (242). Within 120 days after discharge, CRP levels higher than 9.6 mg/L were independently associated with higher all-cause mortality (adjusted HR 1.68) and values between 3.2 to 9.5 mg/L were independently associated with the composite end-point of all-cause mortality and AHF re-hospitalization (adjusted HR 1.31) (242). A larger study with AHF patients (n=4056) concluded that the cumulative 1-year mortality was significantly higher in patients with discharge CRP value greater than 10mg/L compared to lower values (24.1% vs 13.9%, log-rank p<0.001) and even after a multivariable analysis, the mortality risk in the high CRP group remained significant compared to the lower group (HR, 1.43; 95% CI, 1.19 to 1.71; p<0.001), regardless of other clinical factors (243).

Impaired exercise tolerance is a hallmark of HF. A study with symptomatic HF patients of various EF (n=200) demonstrated that CRP levels significantly correlated

with peak oxygen consumption (peakVO_2) in all patients (R = -0.350, p < 0.001) and also separately in the subgroup of HFrEF patients (n= 109) (R = -0.282, p < 0.001) and HFpEF (n=57) (R = -0.459, p < 0.001) (244). Although CRP did not correlate with NT-proBNP (R = 0.05, p = 0.426), it independently predicted peakVO_2 (R = 0.421, p < 0.001) (244). In ischemic HFrEF patients hs-CRP levels correlated with reduced exercise time (R = -0.65, p = 0.006), lower VO_2 at the anaerobic threshold (R = -0.66, p=0.005), and lower peakVO_2 (R=-0.70, p=0.002) (245). Furthermore hs-CRP values significantly correlated with impaired gas exchange (depicted as elevated ventilation/carbon dioxide production slope (R = +0.64, p = 0.008) and reduced end-tidal CO_2 at rest and exercise (R= -0.759, p = 0.001 and R = -0.739, p=0.001, respectively) (245).

In HFpEF CRP has been associated with greater comorbidity burden (like higher body mass index, chronic obstructive pulmonary disease, lower peak oxygen consumption and higher endothelin-1 and aldosterone levels) and some markers of disease severity but it may also be normal in up to 40% of patients (246). A systematic review and meta-analysis with 19 studies demonstrated that CRP predicts the development of HFpEF (HR: 1.08; 95% CI: 1.00-1.16; P = 0.04; I^2 = 22%), the increased risk of CVD when used as a categorical (HR: 2.52; 95% CI: 1.61-3.96; P < 0.0001; I^2 = 19%) or continuous variable (HR: 1.24; 95% CI: 1.04-1.47; P = 0.01; I^2 = 28%), and all-cause mortality (categorical variable HR: 1.78; 95% CI: 1.53-2.06; P < 0.00001; I^2 = 0%, continuous variable HR: 1.06; 95% CI: 1.02-1.06; P = 0.003; I^2 = 61%) (247).

The prognostic role of hs-CRP has been verified in patients with atrial fibrillation (AF) as well (248). A study with subjects diagnosed with AF with or without HF (n=3784) were followed for a median of 4 years. After multivariable adjustment hs-CRP was significantly associated with the risk of HF hospitalization ([HR], 1.22; 95% CI, 1.11-1.34) (248). In a study with 735 hypertensive subjects without HF or CV risk factors and a mean follow-up of 114 months, serum hs-CRP maintained an independent association with HF development in the multiple Cox regression analysis (HR = 1.162, 95% CI = 1.072-1.259) (249). Subjects with hs-CRP

values above the median and forearm blood flow under the median also had increased risk of developing HF (HR = 7.699, 95% CI = 4.407-13.451) (249).

CRP seems a useful biomarker in prognosis; however larger prospective studies are still required to strengthen its role in improving patient outcome.

10. Insulin-like Growth Factor Binding Protein 7 (IGFBP-7)

The insulin-like growth factor binding protein 7 (IGFBP-7) is a secretory glycoprotein expressed in the heart, vascular endothelial cells, fibroblasts and other tissues (250). IGFBP-7 serum concentration has been correlated with functional capacity and prognosis in HF patients (251) and it has been also correlated with echocardiographic structural and functional abnormalities in patients with HFpEF (252). In HFpEF IGFBP-7 was significantly associated with LA dilation (LA size and LA volume index (LAVi) both $p < 0.001$); it was modestly associated with early mitral inflow velocity/annular diastolic velocity ($p=0.03$) and early diastole/peak late diastolic velocity ratio ($p=0.04$) (253). In the acute setting, higher IGFBP-7 levels were associated with increased LAVi ($r = 0.49$, $p < 0.001$), lower LVEF ($r = -0.27$, $p < 0.001$), lower right ventricular fractional area change ($r = -0.31$, $p < 0.001$), and higher tissue Doppler E/e' ratio ($r = 0.44$, $p < 0.001$) (254).

A study that compared HF patients with either preserved or reduced LVEF (n=165) reported that serum IGFBP-7 correlated with NYHA class (HFpEF r=0.25; p=0.020, HFrEF r=0.26; p=0.022), NT-proBNP (HFpEF r= 0.53; p<0.001; HFrEF r= 0.50; p<0.001) and renal function as expressed by e-GFR (HFpEF r= -0.47; p<0.001 HFrEF: r= -0.45; p<0.001) in both groups. Furthermore, IGFBP-7 was higher in HFrEF compared to HFpEF, while in HFpEF IGFBP-7 values correlated with echocardiographic features of diastolic function like E/e´ (r=0.31; p=0.012) and E/A ratio (r=0.31; p=0.011), while in HFrEF IGFBP-7 values correlated with age (r=0.29; p=0.009) and the existence of AF (r=0.34; p=0.002) (250).

In AHF, IGFBP-7 significantly reclassified diagnosis when added to NT-proBNP (net reclassification index: +0.25), while higher concentrations predicted death or re-hospitalization at 6 months (HR 1.84 per $\log_2$-SD; 95% CI: 1.30 to 2.61; $p = 0.001$)

(255). Another study compared the prognostic value of CT-IGFBP-4, NT-proBNP, CRP, and their combinations, in AHF patients (n=156) during a 1-year follow-up The receiver operator characteristic area under the curve (ROC AUC) of CT-IGFBP-4 for all-cause mortality (0.727) was significantly higher than that of the other biomarkers (0.680, P=0.045, 0.669, P=0.016 respectively) while the combination of the three biomarkers predicted mortality significantly better (ROC AUC = 0.788) than any of the biomarkers alone (P<0.01) (256). CT-IGFBP-4 was independently associated with 1-year mortality (HR=3.26, P=0.0008) after adjustment for age, gender, prior HF, CAD, hypertension, CKD, DM, heart rate, haemoglobin, plasma sodium, NT-proBNP, CRP, cystatin C, and elevated TnI or TnT, and it was weekly correlated with NT-proBNP (r= 0.16, P=0.044) (256).

In CHF, a study reported (n=263) that serial IGFBP-7 measurements during a 2.2-year follow up predicted a composite end point of HF hospitalization, cardiac death, LVAD implantation, and heart transplantation [HR (95% CI) per 1-SD increase: 2.45 (1.91 to 3.13] (257). Independently of the biomarker's levels, the annual rate of biomarker change was also significant predictors [per 0.1-SD increase: 1.23 (1.11 to 1.37)] (257). IGFBP-7 levels have been reported to decrease after heart transplantation and this change is correlated with improved mean right atrial pressure (r_s = 0.71; p = 0.00011) and NT-proBNP (r_s = 0.71; p < 0.0001) (258).

In AF patients, higher plasma levels of IGFBP-7 were significantly and independently correlated with HF hospitalization (n=3691) (259). Data from two prospective multicenter observational cohort studies reported that across increasing IGFBP-7 quartiles the multivariable adjusted hazard ratios (aHRs) (95% CI) were 1.0, 1.05 (0.63-1.77), 2.38 (1.50-3.79), and 4.37 (2.72-7.04) (p for trend < 0.001) and in a subgroup of patients without HF at baseline (n=2812) the corresponding aHRs were 1.0, 0.90 (0.47-1.72), 1.69 (0.94-3.04), and 3.48 (1.94-6.24) (p for trend < 0.001) (259). Patients with elevated IGFBP-7 had a higher risk of HF hospitalization (aHR 2.17; 1.30-3.60) (259). Another study with community based elderly subjects (n=2001) reported that IGFBP-7 was independently associated with prevalent AF and echocardiographic variables of diastolic dysfunction (E/e' > 8, LA enlargement, LV hypertrophy and reduced midwall circumference shortening (MFS) after adjusting

for age and sex (260). After adjustment for clinical risk factors IGFBP-7 independently predicted all-cause mortality (HR 2.13[1.08-4.22]) in a median follow-up of 10.6 years (260).

These data show that IGFBP-7 could be used as a potential prognostic HF biomarker, especially combined with NT-proBNP, in order to add prognostic information.

11. Albumin

Low serum albumin is common in HF however its pathophysiology and clinical implications are moderately understood. A meta-analysis of 48 studies with chronic and acute HF patients (n=44.048) reported that hypoalbuminemia was found in 32% of the participants (95% [CI] 28.4%-37.4%) (261). In 10 studies evaluating AHF, both in-hospital and long-term mortality were increased in patients with low albumin ([OR] 3.77, 95% [CI] 1.96-7.23, and [OR]:1.5, 95% CI: 1.36-1.64 respectively) (261). Pooled area under the curve (AUC 0.73; 95% CI 0.67-0.78) was comparable to BNP in predicting mortality in HF patients, suggesting that serum albumin may be useful in determining high-risk patients (261).

A study with 509 AHF subjects reported that hypo-albuminemia was an independent predictor of long-term mortality with values less than 35.7 g/L having a [HR] of 2.01 (95% CI 1.24-3.25, p=0.003) (262), while in another study the combination of increased albumin and bilirubin on admission was independently associated with increased in-hospital mortality ([OR] 6.0, 95% [CI] 1.8-19.6, p=0.0017) (263). On the other hand, there are data that do not associate serum albumin with short-term clinical outcomes in AHF (264).

In CHF, a study with 5779 HF patients and a mean follow-up of 19 months demonstrated that decreasing levels of albumin were significantly associated with mortality (lowest quartile compared to highest: [HR] 5.74, 95% [CI] 4.08-8.07, p<0.001) (265). Cox regression analysis of albumin as a continuous parameter demonstrated that reduced albumin levels were directly associated with increased

mortality (p<0.001 adjusted model). Furthermore decreased albumin significantly predicted HF-related hospitalizations ([HR] 2.58, 95% [CI] 2.12-3.14, p<0.001) (265). Furthermore, albumin-to-globulin ratio has a good prognostic value and constitutes an independent predictor of 90-day and one-year mortality in chronic HFrEF patients (n=999) (266).

In patients with HFpEF, there are more data regarding the prognostic role of serum albumin compared to HFrEF. A study with 372 subjects (enrolled in the TOP-CAT trial) reported that lower albumin was associated with older age, black race, and worse NYHA class, PAD, AF, DM, increased inflammatory biomarkers, albuminuria, and greater arterial stiffness, diastolic dysfunction and pulmonary hypertension (267). Albumin was a strong predictor adverse prognosis (267). In another study serum albumin levels <3.2g/dl were also considered as significant, independent predictors of all-cause mortality (p-value <0.0001) in as tudy with acutely decompensated HFpEF patients (n=427) (268). Serum albumin has also been associated with myocardial fibrosis, adverse pulsatile aortic hemodynamics, and prognosis in HFpEF (267). Hypoalbuminemia is associated with higher myocardial extracellular volume (p=0.0023) and greater NT-proBNP levels, but not with a higher myocardial cellular volume (p=0.13), as well as with an increased forward wave amplitude and pulsatile power in the aorta (267). Serum albumin was a strong predictor of death or HF hospitalization even after adjustment for NT-proBNP levels and the Meta-Analysis Global Group in Chronic Heart Failure (MAGGIC) risk score (adjusted standardized [HR] 0.56; 95% CI=0.37-0.83; p<0.0001) (267).

12. Carbohydrate Antigen-125 (Ca-125)

Carbohydrate antigen-125 (Ca-125) is primarily a biomarker used for the diagnosis of ovarian cancer; it is a glycoprotein synthesized by epithelial cells in response to mechanical (hydrostatic pressures) and/or inflammatory stimuli, which are nevertheless main pathophysiological pathways in HF (269). As such, recent work has investigated its role in risk stratification and monitoring in HF.

A meta-analysis of 16 studies (n= 8401) of subjects with AHF and a mean follow-up of 13 months reported that Ca-125 levels are higher in congestive HF patients compared to euvolemic patients and that they are associated with increased all-cause mortality (8 studies, [HR]: 1.68, 95% [CI] 1.36-2.07; p<0.0001; I^2: 74%) and HF-related readmissions (5 studies, [HR]: 1.77, 95% [CI] 1.22-2.59; p<0.01; I^2: 73%) (270). After an AHF hospitalization, both admission and discharge Ca-125 values predicted mortality, and a decrease in Ca-125 predicted a 68% reduction in the 1-year mortality risk in patients with hospital stay longer than 10 days (271). The combination of high Ca-125 and NT-proBNP values either in admission or in serial measurements can significantly predict mortality (272, 273). Long-term serial kinetics of Ca-125 and NT-proBNP of patients with AHF and a median follow-up of 2.64 years (IQR=1.20-5.36), revealed that the trajectories of both biomarkers markedly correlated with survival status as patients that died had steadily higher values (272). After adjustment for established risk factors and for each other, the baseline absolute change in Ca-125 and NT-proBNP was significantly associated to mortality ([HR]=1.05 (1.01-1.09); p=0.011 (AUC)=0.76) and ([HR]=1.04 (1.02-1.06); p<0.001 (AUC=0.75) respectively (272). The same applies also for AHF patients that also have impaired renal function, where actually the correlation between Ca-125 and survival remained consistent across all eGFR categories (all-cause mortality, P-value for interaction=0.559; CV mortality, P-value for interaction=0.855) while NT-proBNP associated with mortality in patients up to mildly reduced eGFR, with its predictive ability progressively decreasing at lower eGFR (<45mL/min/1.73m^2) (274).

A retrospective study with acutely decompensated HF patients of various LVEF (n=1.387) revealed that length of stay had a differential effect (7 days threshold) on the risk of 6-month AHF readmission, across Ca-125 levels (p=0.01) (275). More specifically, patients with lower Ca-125 values (<35 U/mL) did not modify the risk of AHF hospitalization ([HR]:1.31; 95% [CI]: 0.92-1.87, p=0.131), contrary to those with higher biomarker values where hospitalization for a larger period was associated with a lower risk of AHF readmission ([HR]:0.70; 95% [CI]: 0.51-0.98, p=0.036) (275). In that sense, high Ca-125 levels may identify those patients that could benefit from a prolonged hospitalization in order to reduce the risk of future HF readmissions (275). Additionally Ca-125 is positively correlated with

intra-renal venous flow (IRVF), which constitutes a potential surrogate biomarker of renal congestion and adverse outcomes in HF, and in that aspect, it surpasses NT-proBNP in risk stratification (276). In decompensated HF, Ca-125 was positively associated with parameters of congestion such as NT-proBNP and a composite congestion score (277).

Concerning right-sided heart failure, Ca-125 has been proposed as a useful biomarker, with the five main factors associated with it being, in order of importance, the presence of pleural effusion (36.8%), tricuspid regurgitation (TR) severity (25.1%), age (11.9%), NT-proBNP (6.5%) and peripheral oedema (4.3%) (278, 279). In another retrospective study Ca-125 is positively correlated with RV end-diastolic diameter, pericardial effusion, mean RAP and PAWP, and negatively correlated with 6MWD, LVEDD, mixed venous oxygen saturation, and cardiac index; ROC analysis showed that Ca-125 has additional predictive value to NT-proBNP for the severity and prognosis oif pulmonary hypertension (280). Finally, in patients with TR (n=2.961) multivariate analysis revealed a differential prognostic effect for Ca-125 and NT-proBNP, depending on regurgitation severity ($p<0.05$) (281). NT-proBNP significantly linearly associated with mortality in non-severe TR ($p<0.001$) but not in severe TR ($p= 0.308$), while Ca-125 was significantly associated with mortality risk both in severe ([HR]: 1.09; 95% [CI]:1.03-1.14; $p=0.001$), and non-severe TR ([HR[: 1.28; 98% [CI]:1.11-1.48; $p=0.001$), where it actually outperformed NT-proBNP (281).

In octogenarians, early post-discharge Ca-125 was an independent predictor of all-cause mortality at 1-year ([HR] 1.41; 95% [CI]: 1.21-1.63; $p<0.001$), but not in 30-days (282). In AHFpEF patients older than 70 years old Ca-125 remained positively associated with a higher risk of mortality and re-admissions at one year ($p=0.009$) (283). However, in multivariate analyses, it seems that the biomarker is independently positively related to age ($\beta = 3.93$ for 10 years), which means that its usefulness may be limited (284). Finally, in elderly people with HF, Ca-125 was associated with the presence of permanent AF, with a cut-off level of 91 U/mL, a specificity of 84% and a sensitivity of 54% (285).

In patients undergoing TAVI, Ca-125 levels before and after intervention independently predict death and MACE (death, HF admission, MI, and stroke) (286). Compared to NT-proBNP ([HR] 1.58; p=0.194), high Ca-125 ([HR] 4.62; p=0.001), persisted as an independent predictor of the primary end point (287). Addition of Ca-125 significantly improved the predictive value (C-statistic: 0.805 vs 0.776) and the net reclassification index (50%, 95% [CI]: 20-84) with an integrated discriminative improvement of 3.0% (287). Furthermore, another useful biomarker for HF and for adverse events after TAVI, namely Gal-3, was associated with a higher risk of the primary endpoint only when Ca-125 was elevated (38.8% vs 18.2%, [HR], 2.02; p=0.015) and not when Ca-125 was normal (6.6% vs 6.7%, [HR] 1.16; p=0.981), with a differential prognostic effect of Gal-3 across Ca-125 status (P for interaction=0.048) (287).

Current evidence supports Ca-125 role in risk stratification, monitoring, and guidance of therapy during the first weeks and months after an AHF event; in addition Ca-125 is advantageous from a logistic aspect as it is already widely used, it is independent of the processing time, biologic fluid (plasma or serum), while it has a significantly lower cost than NPs (279).

13. Uric Acid (UA)

Plasma Uric acid (UA) level is the result between food intake, endogenous purine degradation, and the UA excretion which involves the intestine (25% clearance) and kidney (75% renal clearance). Reduced renal excretion of UA is the more prevalent etiology of hyperuricemia in patients with gout, while overproduction seems to be the main reason for elevated UA levels in CVD, as a result of increased oxidative stress. The mechanisms of production of UA can be particularly active in patients with HF, with an over-expression of xanthine oxidase (XO) activity which is associated with impaired LV function (288). Degradation of xanthine to UA by XO generates reactive oxygen species (ROS), which may lead to cardiac dysfunction and also reduced nitroxide (NO) bioavailability, causing

endothelial dysfunction through vasoconstriction which is largely involved in the pathogenesis and clinical profile of CVD including HF (289).

Many studies have found an association between UA levels and HF symptoms according to NYHA class, reduced exercise tolerance, systemic congestion, and decreased cardiac function, mean pulmonary arterial pressure (mPAP), and pulmonary capillary wedge pressure (PCWP) (290-292). Elevated serum uric acid levels are associated with diastolic dysfunction in patients with DCM (293). Hyperuricemia has also been found to be an independent risk factor for HF development in young theoretically healthy subjects (294). Older asymptomatic hyper-uremic subjects (≥65years) in a large registry (n=2749) with a 4-year follow up had an increased cumulative incidence of incident HF events (6.5% vs 3.1%, [OR]=2.15, 95% [CI]: 1.39-3.33, p=0.001), even after adjusting for confounding factors, including baseline eGFR (HR = 2.34, 95% CI: 1.50-3.63, p<0.001) (295). Current evidence suggests that UA could be both an indicator of poor prognosis and a potential determinant in the pathogenesis of HF (296). Two recent studies in patients hospitalized for AHF found a strong association between increased UA levels on admission and the companied risk of all-cause mortality and/or HF re-hospitalization during the first 30 days post discharge and during a 6-month follow-up period respectively (297, 298). Combined hyperuricemia and high pulse pressure was found to improve the risk stratification in the prediction of incident HF in an elderly population of 1665 patients from Taiwan (299).

More recently, the prognostic value of elevated UA in the clinical practice of HF was assessed by a post-hoc analysis of the MECKI (Metabolic Exercise Cardiac Kidney Index) score database, which includes a large optimally treated HFrEF patient population. UA was associated with both CV and all-cause deaths also in the HFrEF population, but only in patients with NYHA class I–II (300). UA is more strongly associated with death in patients with more preserved exercise performance, and when added to the MECKI score, it did not add prognostic power either in the general population or in HF patients (300). However, in a recent study UA levels were independently a better predictor of poor outcome than NT pro-BNP in HF patients (301).

Concerning the impact of allopurinol – a UA lowering treatment – on all cause and CV mortality the results are conflicting. There are studies where allopurinol has been associated with a decreased CV risk among hyperuricemic patients (302) but there is also data for the opposite. In a meta-analysis with high heterogeneity (heterogeneity x^2=37.3, I2=73%, p<0.001) allopurinol treatment was associated with a significant increase in the risk for all-cause mortality (HR: 1.24, 95% CI: 1.04-1.49, p=0.02). Concerning CV mortality, allopurinol treatment was associated with a 42% increased risk of CV mortality (HR: 1.42, 95% CI: 1.11-1.81, p=0.005). There was also a trend toward increased CV hospitalization in the same group (303). Finally, there are studies resulting in a neutral effect from the use of allopurinol in high-risk HFrEF patients regarding clinical status, exercise capacity, quality of life, or LVEF at 24 weeks (304).

Angiotensin receptor-neprilysin inhibitor (ARNI), another UA lowering medication was also studied in 4795 HFpEF patients with increased uric acid levels at baseline and 4 months after treatment initiation (305). After multivariable adjustment, hyperuricaemia was associated with increased risk for the primary outcome (cardiovascular death and total HF hospitalizations) [rate ratio 1.61, 95% confidence interval (CI) 1.37-1.90], while initiation of ARNI treatment reduced UA levels by 0.38 mg/dL (95% CI 0.31-0.45) (305). The effect was greater in those with elevated UA prior to treatment. After multivariable adjustment, change in UA was directly associated with high-sensitivity troponin T (p<0.05), and UA at 4months was a stronger predictor of adverse outcomes than baseline UA (305).

Raised UA values are common in HF and, as mentioned above, they are prognostic of increased morbidity and mortality. There is robust data that support the hypothesis that UA is a reliable marker, but that does not necessarily mean that UA has an active role in the pathogenesis of HF (291). Whether UA constitutes a therapeutic target is still to be proved. Future and ongoing clinical trials will show if allopurinol or other UA lowering medications will alter the natural course and improve HF outcomes (291).

14. Midregional pro-adrenomedullin (MR-proADM)

Adrenomedullin is present in cardiac, vascular, renal, pulmonary, and endocrine tissues, and has diuretic, natriuretic and vasodilatory properties (306). Its excretion is stimulated by volume overload as a mechanism to maintain endothelial barrier function, preventing thus a vascular leakage that leads to systemic and pulmonary oedema (307). Futhermore, it inhibits the renin-angiotensin-aldosterone system (307). The circulating bio-active molecule in plasma is a potential marker of congestion status both at admission, during and after hospitalization (308, 309), while it has been associated to biventricular pressures recorded during right heart catheterization (310). However, it is difficult to measure due to its short plasma half-life; an inactive, more stable pro-hormone fragment called midregional pro-adrenomedullin (MR-proADM) can be quantified instead.

In AHF, one study with 917 patients with HFrEF demonstrated that MR-proADM had superior prognostic significance compared to NPs, (concordance index 0.72 for all-cause mortality), improved Cox regression models including NPs ($P<0.001$), and was the only biomarker also predicting non-cardiac death (HR 1.8 vs. 1.0) in a 18-month follow-up (311). Furthermore six month MR-proADM enhanced models including baseline MR-proADM ($P<0.001$) for prediction of all-cause death (net reclassification index: 0.48, 95% confidence interval 0.19–0.78) (311). Another study with 180 AHF patients found that MR-proADM had the highest area under the curve (AUC) for death during the first year compared to MR-proANP and NT-proBNP while the NPs had a higher AUC after 1 year. The biomarker was independently prognostic and reclassified risk at 1 year [HR = 2.70; $p < 0.001$] and at 4 years [MR-proADM HR = 1.51, $p = 0.03$] (312). In the MOCA study with acute HF patients, MR-proADM provided incremental value for risk stratification at 30-days and one year (313). In the Emergency Department (ED) setting, the value of bioactive adrenomedullin of patients with AHF was strongly predictive (adjusted odds ratio per IQR change: 2.68; 95% CI: 1.60, 4.51) of clinically important 30-day outcomes (all cause mortality, cardiac arrest with resuscitation, respiratory failure, emergency

dialysis, ACS, hospitalization >5 days, and repeat ED visit or hospitalization) after adjusting for biomarkers like BNP, TnI, creatinine, and sodium (314). In as study with 1236 AHF hospitalized patients, higher discharge bio-ADM levels were combined with higher use of loop diuretics (reflecting residual congestion) and were independently associated with a greater risk of 60-day HF re-hospitalization (HR 4.02, 95% CI 2.23-7.26; p <0.001) (315).

In patients with CHF MR-proADM levels increase with NYHA class (p< 0.0001) and also predict survival at 12 months (HR 1.82, 95% CI 1.24-2.66, p = 0.002) after multivariable adjustment (316). In chronic HFrEF patients (n=786) MR-proADM levels predicted mortality at 2 years [HR = 1.77, p < 0.001] in addition to age (HR = 1.02, p = 0.004), EF (HR = 0.98, p = 0.004), and NYHA class > II (HR = 1.86, p< 0.001) (317). A study with stable CHF patients (n=724) followed-up for 6 years showed that MR-proADM had a predictive value for all-cause mortality which was independent of BNP, echocardiographic features, clinical parameters of mortality, and the Framingham risk score (HR 3.78, p<0.001 for stage A and HR 2.744, p<0.001 for stage C/D respectively, with a trend for stage B patients HR 1.579, p=0.05005) (318).

A serious advantage of MR-proADM compared to other HF biomarkers, is that unlike NPs, MR-proADM (and also GDF-15) was not influenced by BMI when studied in obese HF individuals (319). Moreover, according to recent data MR-proADM outperformed NT-proBNP for the diagnosis of AHF in the presence of AF, with little change in performance (AUC 0.83 in non-AF vs. 0.76 in AF) compared to NT-proBNP (0.91 vs. 0.71) and hs-cTnT (0.83 vs. 0.62) respectively (320). The accuracy of MR-proADM (73.3%) for diagnosing AHF among patients with AF was superior to both NT-proBNP (61.6%) and hs-cTnT (64.6%) (321).

15. Cystatin C (CysC)

Cystatin C is a serum protein related to renal function that appears to be independent of age, sex, and muscle mass (321, 322). It is directly associated, in a dose-response manner, with a higher risk of death from all causes and cardiovascular events and constitutes a stronger predictor than creatinine in elderly people (321),

without having a causal role in CVD etiology as shown by a large multicenter study that incorporated data from 16 prospective cohorts (n = 76.481) with 37.126 measures of cystatin C and 63.292 CVD events (323). Despite the fact that Cystatin C is mainly a biomarker used for renal dysfunction, the association with HF remains significant in patients with normal GFR levels (324).

A meta-analysis of 10 prospective studies of HF patients (n=3155) indicated that cystatin C was associated with an increased risk of all-cause mortality (HR: 2.33; 95% CI:1.67-3.27; p<0.001) but not with a combined endpoint of mortality/rehospitalization (HR: 2.06; 95%CI: 1.58-2.69; p=0.181) (325).

In AHF, a RCT (n=811) reported that higher baseline (but not follow-up) Cystatin C levels were associated with increased risk of 30-day adverse events, less improvement in dyspnea after 24h, and increased 180-day mortality (326). Another study with patients presenting with AHF (n=207) reported that in multivariable regression analysis cystatin C predicted mortality after the adjustment for renal function, AKI, BNP and HF risk factors (HR 1.41; 95% CI 1.02-1.95) (327). A Swedish cohort with hospitalized AHF patients (n=286) reported that circulating cystatin C predicted mortality after discharge (HR 2.11, 95% CI 1.56-2.86; (p=0.0000001) better than other biomarkers (MR-proADM, copeptin, C-terminal pro-endothelin-1, NT-proBNP) (328). Other studies reported that either cystatin C could be better prognostic biomarker for the composite endpoint of cardiac death or recurrent HF compared to UA and NT-proBNP, over a two-year period after AHF (329), or that the combination of NT-proBNP with estimated GFR using cystatin C equations better predicts outcomes than either parameter alone and additive prognostic value (330). Although in patients hospitalized with AHF, serum creatinine- and cystatin C-based predictions performed poorly in detecting actual changes of GFR (331), the estimated GFRs calculated with cystatin C predicted mortality more accurately than the creatinine derived eGFR (332, 333).

Cystatin C is positively correlated with 5-year all-cause mortality in CHF patients, along with cardiac function and NT-proBNP (334). The levels of cystatin C correlate positively with NYHA class, major adverse cardiovascular events (MACE),

left ventricular end diastolic diameter (LVEDD), and negatively with LVEF (all $p<0.05$) (335). Taking echocardiographic features into account, in patients with HFpEF due to arterial hypertension, cystatin C is associated with diastolic dysfunction and alterations in collagen metabolism independently of eGFR, and it might contribute to diastolic dysfunction by facilitating myocardial fibrosis via accumulation of osteopontin and TIMP-1, the inhibitor of matrix metalloproteinase-1(MMP-1) (336). In HFrEF circulating cystatin C is positively correlated with LAVi (R^2 =0.135, p=0.019) (337).

In patients receiving advanced HF therapies, cystatin C retains its prognostic value. In 92 HFrEF patients that received CRT, cystatin C levels measured at implantation and 1 month; those with high values after 1 month had more than increased risk of CRT non-response (OR ratio uncompensated 9.0; compensated 36.4; both $p\leq0.004$) and MACE (HR uncompensated 8.5; p=0.005) (338). Among patients with LVAD (n=116) the eGFR estimated by cystatin C predicted the primary end point (composite of in-hospital mortality, renal replacement therapy, or severe RV failure) compared to creatinin eGFR that did not [odds ratio per 5 mL/min/1.73 m^2 decrease 1.16 (1.02-1.31) vs 0.99 (0.94-1.05)] (339). In patients undergoing percutaneous mitral valve repair (PMVR - MitraClip) (n=120), baseline levels of serum cystatin C were higher in those that died within 12 months compared to those that survived and had a significant reduction in MR severity ($p<0.001$) and NYHA class improvement ($p<0.01$) (nonsurvivors: 2.4 mg/L [interquartile, IQR: 1.7;3.1] vs survivors: 1.7 mg/L [IQR: 1,3;2.1], $p<0.001$) (340).

On the contrary, in a large meta-analysis with data from 4 community-based cohorts with an average follow-up of 12.5 years, cystatin C was significantly associated with development of HF in both sexes, however when further adjusted for NPs the association was not significant (341).

Cystatin C is a useful biomarker for prognosis both in acute HF but also in chronic as well, with its main advantage being the fact that it predicts renal function independently of body mass which is reduced in HF patients.

16. Cyr61-cysteine-rich protein 61 (CCN1/CYR61)

Cyr61-cysteine-rich protein 61 (CCN1) is a heparin-binding protein, that integrates its activities on macrophages, endothelial cells, vascular cells and fibroblasts to regulate angiogenesis, inflammation and matrix remodeling (342). Myocardial expression of CCN1 can be induced by pressure overload and neurohormonal factors, such as α1-adrenergic stimuli, which indicates that CCN1 could have an important role in the adaptation of the heart to cardiovascular stress (342). A prospective observational study in 113 AHF patients with established CAD showed that CCN1 was a significant predictor of 30-day all cause mortality independently from other established markers (NT-proBNP, cTnI, eGFR, hemoglobulin etc) (342).

A second study found that serum CCN1 in AHF patients (n=183) was significantly increased compared to controls (237 pg/ml vs. 124.8 pg/ml, $p< 0.001$) and although moderately associated with the level of NT-proBNP ($r=0.349$, $p< 0.001$) it was not affected by LVEF, eGFR, age or atrial fibrillation (341). Kaplan-Meier curve analysis illustrated that AHF patients with CCN1 level > 260 pg/ml had lower 6-month survival ($p< 0.001$) while multivariate Cox hazard analysis suggested that CCN1 is an independent predictor of 6-month all cause mortality in AHF (HR 5.825, 95% CI: 1.828-18.566, $p=0.003$). When combined with NT-proBNP CCN1 significantly improved the C-statistic for predicting death (0.758, $p<0.001$) (343).

This data suggests that CCN1 is a promising prognostic biomarker for AHF patients.

17. Endotrophin

Endotrophin is a profibrotic by-product of collagen VI which is a triple-helix molecule composed of A1, A2 and A3 polypeptide chains secreted from connective tissue, especially from adipose tissue (344). Type VI collagen is associated with many fibrotic diseases such as hepatic, renal, pulmonary and cardiac fibrosis. Endotrophin is a newly identified adipokine that is a soluble proteolytic fragment of the A3 chain of type VI collagen (345), and one study investigated its role in the complex process

of HF fibrosis, and its relationship with the renin-angiotensin-aldosterone system (RAAS) blockade in patients with EF<50% compared to controls (346). Although there was no significant difference for endotrophin levels between the patient and control group (p=0.35), the endotrophin levels were significantly lower in HFmrEF patients (p=0.03) using RAAS blockers, suggesting that RAAS blockers may influence endotrophin levels and thus could have a role in the prevention of remodeling taking place in HF (346).

18. Non coding (circular and micro) RNAs

Circular RNAs (circRNAs) are non protein coding RNAs that are located in the nucleus and cytoplasm. Most circRNAs contain two to three exons, but some genes [like titin and ryanodine receptor 2 (RYR2)] produce many different circRNAs derived from multiple exons or introns (347). CircRNAs have been detected in blood and therefore raised the possibility that they might constitute a reservoir of novel biomarkers and thus a potential diagnostic and therapeutic tool for HF management (347). However, only a small number of circRNAs have been statistically promising so far, namely cTTN1, cTTN2, cTTN4, CTTN5 (348) and MICRA (349).

The first circRNA that was described to be functional in the heart was termed heart-related circRNA (HRCR). It is normally present in mouse hearts and it is repressed in hypertrophic and failing hearts (350). HRCR binds and thereby sequesters miR-223, a micro RNA that causes cardiac hypertrophy via inhibition of the protein ARC (apoptosis inhibitor with CARD domain).

A second circRNA that may regulate cardiac ageing is circ-Foxo3 (351). Functionally, circ-Foxo3 binds to several proteins known to be involved in cellular stress response, like inhibitor of DNA binding 1, E2F transcription factor 1, hypoxia-inducible factor 1α, and focal adhesion kinase, resulting in cytoplasmic sequestration of these proteins (347). Whether circ-Foxo3 contributes to cardiac ageing in vivo remains to be established (351).

CircRNAs in the vasculature that could potentially contribute to HF have also been described; most notably cZNF292 which is expressed in the endothelium and induced by hypoxia, thus meaning that it may be increased in the hypoxic myocardium (352).

Micro RNAs (miRNAs) are also other non coding RNAs that showed promising results as novel HF biomarkers. The most widely studied was miR-423-5p. In acute HF patients miR-423-5p has been associated with worsening renal function (353). In chronic stable HF patients miR-423-5p has been associated with diagnosis (90% sensitivity and specificity) and also it is correlated wiith prognostic features such as elevated NPs, QRS interval broadening and left chamber dilation (354). In a small study (n=62) circulating miR-21-5p, miR-30a-3p, miR-30a-5p, miR-155-5p, miR-216a and miR-217 were expressed differently among healthy controls and HF patients and were unaffected by hemolysis, suggesting that they may be used potential biomarkers in HF (355).

Non coding RNAs are a promising tool for HF management but the molecular mechanisms through which they regulate gene expression and HF developments are poorly characterized (347) and further research is still needed.

19. Matrix metalloproteinases (MMPs)

The myocardial extracellular matrix (ECM) is a dynamic environment that regulates and protects the structural and physiological homeostasis of the heart, and modifications in ECM may lead to diastolic or systolic dysfunction and HF development (356). Matrix metalloproteinases (MMPs) regulate the degradation of ECM and lead to myocardial remodeling that takes place in HF, and may constitute potential biomarkers for the identification of patients at risk for HF development, their stratification and prognosis. Their activity is controlled by regulation of expression and proteolytic activation of pro-enzymes and by the Tissue Inhibitors of Metalloproteinases (TIMPs) and the two most studied MMPs are MMP-2 and MMP-9 (357). A study with CHF patients (n=173) and a median follow up of almost 9 years found that MMP-9 levels were higher in patients with HF events (P = 0.004), with a cut-off value of 23.2 ng/mL (p=0.005) (357). The MMP-9/TIMP-1 ratio increased with increasing NYHA class, while multivariate Cox proportional hazard model showed that high MMP-9 values were an independent predictor of HF events ([HR] 3.73; 95% [CI] 1.03-13.46; p=0.043) (357). In a study with HFrEF patients that underwent CMR, MMP-9 levels were significantly higher in patients with cardiac fibrosis (n=32) than

those without fibrosis (n=21) (p<0.01) while furthermore there was a statistically significant correlation between MMP-9 levels and LVEDV and LVESV (358).

In a Japanese study with elderly subjects (n=370, median age 69 years), serum MMP-2 levels were higher in cachectic patients patients (797.5 [649.0-1066.8] vs 610.0 [461.8-756.8] ng/mL; p=0.004), which had greater values of pulmonary vascular resistance (PVR) (161.9 [119.4-225.4] vs 127.8 [90.7-164.8] dynes/sec/cm^{-5} , p=0.020) and higher probabilities of all-cause death (log-rank P=0.010) compared to non-cachectic subjects (359). Cachexia, which is very common in HF, was reported as an independent predictor of mortality ([HR] 1.89; 95% [CI] 1.06-3.37; p=0.029) (359). A study that compared plasma levels of MMP-2 and MMP-9 in all LVEF spectrum of HF (n=113) showed that for HFpEF diagnosis the AUC of MMP-2 and MMP-9 was 0.705 and 0.597 respectively, for HFmrEF diagnosis the AUC of plasma MMP-2 and MMP-9 was 0.678 and 0.676 and for HFrEF, the AUC was 0.814 and 0.774 respectively (360). Logistic regression analysis showed that MMP-2 but not MMP-9 was independent risk factor for HF (OR = 0.805) (360).

In a Swedish registry with elderly subjects (n=1586, median age>70 years MMP-12 was associated with incident HF after adjustment for established risk factors with worsened echocardiographic LV systolic function at baseline (p<0.02 for all) (361).

Nevertheless, a study with patients with advanced HFrEF (n=18, LVEF=22 ± 2**%**) investigated the relationship between serum MMP-9 and TIMP-1 and cardiac release (362). The trans-cardiac MMP-9 gradient was significantly lower in HF patients (p<0.05), and was negatively correlated with left ventricular mass (r = -0.51, p=0.01), pointing that the circulating levels of MMP-9 and TIMP-1 do not accurately reflect cardiac turnover and further data are needed for the establishment of MMPs as a solid biomarker in HF (362). Finally, a recent study demonstrated that TIMP-1 levels indicated a U-shaped, concaved, non-linear association with all-cause mortality (P for effect = 0.011, P for non-linearity = 0.022) in HF, but no such association for MMP-9 (363).

20. Chloride

Hypochloraemia (<96 mmol/L) is a common laboratory finding in patients with HF and is associated with increased mortality, independently of other prognostic markers like NT-proBNP (364, 365). However, it is not clear whether low serum chloride has a direct pathological effect, it is an adverse effect of diuretic therapy or it is a marker of congestion and disease progression (366) and serum chloride is the least studied major electrolyte form those that are associated with cardiorenal and neurohormonal systems (367). Up to date there are several proposed pathways by which hypochloraemia leads to an adverse outcome in patients with HF: by increasing renin release; by a stimulatory effect on the with-no-lysine kinases which might increase renal sodium-chloride co-transporter activity; and by an adverse effect on myocardial conduction and contractility (368).

In a study with hospitalized AHF patients (n=1.318) admission serum chloride levels were independently and inversely associated with long-term mortality ([HR] per unit change: 0.94; 95% [CI]: 0.92 to 0.95; p<0.001) (369). After multivariable risk adjustment, admission chloride levels remained independently associated with mortality (HR per unit change: 0.93; 95% CI: 0.90 to 0.97; p<0.001) in contrast to admission sodium levels, which were no longer significant (p > 0.05) (369). Another study with 5613 HF patients reported that during admission patients in the first quartile (median chloride 96 mmol/L) were in higher NYHA class, were more likely to take loop diuretics and had a two-fold increased risk of death compared to patients in the fourth quartile (median chloride 106 mmol/L) (p<0.001 for all) (364).

Apart from hypochloraemia on admission, low serum levels on discharge have also been associated with greater all-cause mortality [(HR) 1.44, 95% [CI] 1.15-1.79; p=0.001), all-cause mortality or all-cause readmission (HR 1.26, 95% CI 1.04-1.53; p=0.02), and all-cause mortality or HF readmission (HR 1.41, 95% CI 1.14-1.74; p=0.002) after multivariable adjustment, in 1002 AHF patients with a median follow-up of 28.5 months (370). In 1241 patients hospitalized for AHF, low admission or discharge serum chloride was significantly associated with higher 30-day hospital readmission rate or death (42.2% vs. 33.7%, p=0.008, even after multivariate analysis

for serum sodium, weight loss, diuretic dose, adjunct thiazide use, serum blood urea nitrogen, and BNP levels (OR: 1.35, 95% CI: 1.02-1.77, p=0.033) (371). The kinetics of biomarker during hospitalization could predict HF death in AHF patients, as subjects with persistent hypochloremia (HR, 9.13; 95% CI: 2.56-32.55) and with progressive hypochloremia (HR, 4.65; 95% CI: 1.61-13.4) had a significantly greater risk of HF death than those without hypochloremia during hospitalization (372).

Another interesting study reported that incorporating hypochloremia along with BNP into the preexisting risk model Get With The Guidelines–Heart Failure (GWTG-HF) improved the model performance, with a net reclassification improvement of 0.491 ($p<0.01$) and a good calibration (Hosmer-Lemeshow test: $p = 0.479$) (373).

21. Other biomarkers

Apart from the plasma biomarkers mentioned so far, there is a plethora of other less studied, yet promising biomarkers that could be used in a multi-factorial disease like HF. These molecules have been studied mainly in HFpEF; some of these are:

a) Tetranectin

Two studies have measured tetranectin (gene code CLEC3B), a calcium-binding homotrimeric protein that participates in tissue remodeling by binding to extracellular matrix components like plasminogen and fibrin and regulating proteolysis, with a suggested protective function for the muscle and circulatory system (374). The first study compared tetranectin levels in HFpEF patients (n=40) and controls and found it significantly reduced in HF patients ($p < 0.0001$) (374). Furthermore, tetranectin was associated with HF more closely than BNP (AUC = 0.97 vs 0.84, $p = 0.011$). (374). The second study found that higher concentrations of tetranectin were associated with a lower risk of HF ([HR] 0.82 per 1-SD increase; 95% [CI] 0.71–0.95; $p=6.6\times10^{-3}$) and that this biomarker was significantly associated with all-cause mortality in a multimarker model in 3523 Framingham Heart Study participants (375).

b) Neprilysin

Neprilysin is a cell membrane bound metalloendopeptidase that mediates the anti-fibrotic, anti-proliferative, myocardial relaxation, vasodilator and diuretic properties of NPs; it removes about 50% of circulating NPs (376). Neprilysin inhibition results in increased plasma ANP, urinary cGMP and urinary sodium and reduced LV filling pressures (377). In HFrEF elevated neprilysin levels are associated with increased risk of CV death while its inhibition with sacubitril/valsartan has improved survival (378). A study with symptomatic HFpEF patients (n=242) compared their circulating neprilysin levels with controls and found them lower in HFpEF compared with controls (3.5 ng/ml; [CI]: 2.5 to 4.8 vs. 8.5 ng/ml; CI: 7.2 to 10.0; $p < 0.001$) (379). After adjustment for age, gender, BMI, and smoking history, mean neprilysin levels remained lower in HFpEF (4.0 ng/ml [CI]: 2.7 to 5.4 vs. 8.2 ng/ml [CI]: 6.8 to 9.7; p=0.002) (379).

Another study with dialysis patients (n=153) found that neprilysin activity but not neprilysin concentration was significantly lower in HF vs. controls (0.189 [0.223] vs. 0.257 [0.166] nmol/mL/min, $p<0.001$) and (315 [107] vs. 318 [124] pg/mL, p=0.818) respectively, adding diagnostic information over clinical covariates and NT-proBNP and may aid in diagnosing HF in dialysis patients (380).

c) Pentraxin 3

Pentraxin 3 (PTX3) is a member of the pentraxin family along with CRP and serum amyloid P. It is produced in several tissues but mainly in the vasculature, in response to inflammatory stimuli (381). In stable HFpEF patients (n=360), high plasma levels (>3.0 ng/mL) were significantly associated with CV events (death, nonfatal MI, unstable angina, nonfatal ischemic stroke, HF hospitalization, coronary revascularization) in a mean 30-month follow-up ([HR] 1.16; 95% CI: 1.05 to 1.27; $p<0.01$) (382). The C-statistics for CV events substantially increased from 0.617 to 0.683 when PTX3 was added to five previously identified prognostic factors (i.e age, DM, HF hospitalization, NYHA class, LVEF) (382).

d) Von Willenbrand factor

Von Willenbrand factor (vWF) is a glycoprotein predominantly produced in endothelial cells and mediates platelet adhesion and aggregation, with an important role in hemostasis; increased levels are found in endothelial damage and therefore it is considered as a biomarker of endothelial dysfunction (383). In as study with 457 HFpEF patients vWF levels significantly predicted mortality ([HR] 1.45 per increase of 1-SD, [CI] 95% 1.26–1.68; p<0.001) even after adjustment for age, sex, BMI, NT-proBNP, renal function, and frequent HFpEF-related comorbidities (adjusted [HR]:1.22 per 1-SD, 95% [CI]: 1.05–1.42; p=0.001) (384). Furthermore, vWF had additional prognostic value to NT-proBNP (C-Statistic vWF×NT-proBNP: 0.65 vs NT-proBNP: 0.63; p=0.004) suggesting that it may constitute an independent predictor of long-term outcome in HFpEF subjects, which may be attributed to the endothelial dysfunction as a potential mediator in the pathophysiology of the disease (384).

e) High-density lipoprotein particle subfractions

High-density lipoprotein particle (HDL-P) subfractions affect atherogenesis, inflammation, and endothelial function, factors directly implicated in HF pathophysiology. A study compared patients with HFrEF (n=782), HFpEF (n= 1.004), and controls (n=4.742) and found that mean HDL-P was higher in HFrEF than HFpEF, both of which were higher than controls (all p<0.0001) (385). In both HFrEF and HFpEF, HDL-P was inversely associated with time to adverse events after adjustment for clinical covariates (like HDL cholesterol content, CAD, the inflammatory biomarker GlycA etc) and in sensitivity analyses featuring alternate LVEF definitions, or more strict inclusion criteria with diastolic dysfunction or LVED pressure thresholds (385).

f) Apolipoprotein M

Apolipoprotein M is a lipocalin primarily secreted by the liver. It is present in 5% of HDL and 2% of LDL particles and has anti-inflammatory, antioxidant and anti-atherogenic effects, promoting endothelial protection (386). A study of 297 HF

patients reported that the biomarker was inversely associated with the risk of death ([HR] 0.78, 95% [CI] 0.69-0.88; p<0.0001) and the composite of death/LVAD implantation/heart transplantation ([HR] 0.85, 95% [CI] 0.76-0.94]; p<0.0001), both in HFrEF and HFpEF (386).

g) Soluble CD14

CD14 is a membrane glycoprotein that plays a key role in inflammation and is expressed mostly by myeloid cells. A study with 5217 participants, of which 1878 had HF reported that sCD14 was significantly associated with incident HF ([HR]: 1.56 per doubling, 95% [CI]: 1.29-1.89) after adjusting for clinical and laboratory covariates but this association was significant only for HFpEF ([HR]: 1.50, 95% [CI]: 1.07-2.10) but not HFrEF ([HR]: 0.99, 95% [CI]: 0.67-1.49) (387).

h) Glyc-A

GlycA is a nuclear magnetic resonance composite marker of systemic inflammation. In a study with 6507 participants and a median follow-up of 14 years, subjects in the highest quartile of GlycA experienced increased risk of developing HFpEF ([HR] 2.18, 95% [CI]: 1.15-4.13) compared to those with the lowest quartile, in fully adjusted models, while there was no correlation with developing HFrEF ([HR]: 1.06, 95% [CI] 0.63-1.79) (388)**.**

i) Multiple biomarker panels: LRG1, zinc-alpha-2-glycoprotein, serum paraoxanse/arylesterase and APOA1

The need of identifying better biomarkers for an early HF diagnosis with greater accuracy led to the design of robust mass spectrometry (MS)-based assays for the simultaneous measurement of many potential biomarkers. A recent study applied multiple reaction monitoring (MRM) MS assays to serum samples from HF patients (n=500) (389). Four proteins were significantly differentially expressed between controls and HF subjects (HFrEF and HFpEF); Leucine-rich-alpha-2-glycoprotein (LRG1, p<0.001), zinc-alpha-2-glycoprotein (p=0.005), serum paraoxanse/arylesterase (p=0.013), and Apolipoprotein A1 (APOA1) (p=0.038) (389).

When added to BNP these biomarkers predicted HF with 83.17% accuracy and an AUC of 0.90, compared to 77.1% accuracy and an AUC of 0.86 (p=0.005) for BNP alone (387). Furthermore, LRG1 peptides contributed most significantly to model performance and was significantly associated with future new onset HF [Peptide 1: OR 2.345 95% CI 1.456–3.775, p=0.000; peptide 2: OR 2.264 95% CI 1.422–3.605, p=0.001) (389). This study reported some very promising biomarkers for HF diagnosis and risk stratification that needs to be further assessed.

The most studied serum biomarkers that have shown a potential role in HF management are presented in ***Table 2.***

Table 2. Serum biomarkers that have shown a potential role in HF management

Biomarker	Clinical Usefulness	References (No)
Natriuretic peptides	Diagnosis	(1), (4), (61-62)
	Prognosis	(70-98)
	Therapy monitoring	(99-110)
Troponin	Assists in diagnosis	(4), (62)
	Prognosis	(118-143)
Gal-3	Diagnosis	(62), (155)
	Prognosis	(145-158)
	Therapy monitoring	(160)
ST2	Diagnosis	(165)
	Prognosis	(161-170),(173)
	Therapy monitoring	(171-172), (468)
FGF-23	Prognosis	(175-179), (181)
Copeptin	Prognosis	(187-196)
GDF-15	Diagnosis	(211-212), (214)
	Prognosis	(203-210), (215)
ET-1	Diagnosis	(218)
	Prognosis	(219-223), (225-233)
CRP	Prognosis	(236-250)

IGFBP-7	Prognosis	(251-252), (256-258), (260-261)
	Potential Diagnosis	(119)
Albumin	Prognosis	(262-269)
Ca-125	Prognosis	(271-277), (282-284), (287-288)
	Therapy monitoring	(465-467)
UA	Prognosis	(295-301)
MR-proADM	Prognosis	(312-319)
	Diagnosis (in AF)	(321)
	Therapy monitoring	(468)
Cystatin C	Prognosis	(326-336), (339-342)
CCN1/CYR61	Prognosis	(343-346)
Endotrophin	Therapy monitoring	(347)
Non coding RNAs (circ and micro)	Diagnosis	(119), (348), (355)
	Prognosis	(354-355)
	Therapy targets	(348)
MMPs	Support diagnosis	(119), (358), (361)
	Prognosis	(358-362), (364)
Tetranectin	Prognosis	(376)
Neprilysin	Diagnosis in RD	(381)
	Prognosis	(379)
Pentraxin 3	Prognosis	(383)
vWF	Prognosis	(385)
HDL-P	Prognosis	(386)
Apolipoprotein M	Prognosis	(387)
Soluble CD14	Prognosis	(388)
GlycA	Prognosis	(389)
LRG1	Prognosis	(390)
Chloride	Prognosis	(365-367), (370-373)

RD=Renal dialysis patients

SALIVA BIOMARKERS

Saliva contains several proteins that can be detected and could potentially be used as biomarkers, while the fact that it can be easily collected with non-invasive procedures is a major benefit (3). Furthermore salivary diagnostics is currently catching onto the emerging field of Lab-on-Chip (LoC) and Point-of-Care (PoC) devices (3). On the other hand, despite the fact that collection procedures are easy and painless, saliva composition is affected by several variables, arising from variation of salivary flow rate, circadian rhythm, type of salivary stimulus, diet, age, physiological status, and methods of collection (390) and there is an obvious lack of standardized protocols for biomarker determination in general, and in cardiovascular diseases and HF more specifically. Another reason for the limited use of saliva diagnostics is the fact that levels of most biomarkers are lower in saliva than those found in serum (3). Advances in analytical techniques are expected to make it feasible to measure multiple biomarkers using small volumes of biological fluids including saliva.

Eighteen salivary biomarkers were analyzed for HF; salivary amylase and its major form salivary alpha amylase (sAA), uric acid (UA), 8-isoprostaglandin F2_ (8-isoPGF2_), lactate, galectin-3 (Gal-3), BNP, interleukin 6 (IL-6) and interleukin 10 (IL-10), CRP, protein S100-A7 (S10A7), cortisol, NT-proBNP, 8-epiprostaglandin F2_ (8-epiPGF2_), endothelin, sodium, chloride, and potassium (3, 391-404).

Most of the publications on saliva biomarkers in HF have been conducted during the last decade. The biomarkers have been tested in HF patients and compared to controls and/or different NYHA class patients. The age of participating patients ranged from 39 to 88 years old. Out of the 15 publications in the field, seven included patients with HFrEF only, five with both HFpEF and HFrEF patients (without proving data separately for each phenotype), while three studies did not provide that information. Eight publications included patients with functional class NYHA I, 10 publications included NYHA II, 11 included NYHA III and five included NYHA IV patients. Most patients belonged to NYHA III group, while in four studies the functional class of HF patients was not mentioned (3).

In most of the studies biomarker levels between HF patients and controls were compared, and differences were found to be statistically significant. One study included only HF patients (no controls) and studied the correlation between serum and salivary concentrations for specific biomarkers, with only a weak correlation for serum and salivary IL-6, and no correlation for serum–salivary BNP and IL-10 (394).

Interestingly, although natriuretic peptides have been officially incorporated in the diagnostic algorithm of HF (1), there are only three publications measuring salivary natriuretic peptides (394-395, 400). The first study compared salivary NT-proBNP between controls (n = 40) and HF patients (n = 45). The salivary NT-proBNP concentrations from the healthy participants were below the limit of detection (LOD 16 pg/mL), while in HF patients salivary NT-proBNP ranged from 18.3 to 748.7pg/mL, with a median value of 76.8pg/mL. The salivary NT-proBNP immunoassay showed sensitivity of 82.2% and specificity of 100%, positive predictive value of 100% and negative predictive value of 83.3%, with overall diagnostic accuracy at 90.6% (400). The second study measured serum and salivary BNP from 75 hospitalized patients with HF; no significant correlations were found for serum and salivary BNP ($r = -0.064$, $p = 0.628$), with a large positive bias of 480 pg/mL, indicating that serum concentrations of BNP were much higher than the salivary concentrations, and as serum BNP levels increased, the difference became larger (394). In the third study, mean salivary BNP levels were higher in HF patients, either hospitalized ($p < 0.001$) or outpatients ($p = 0.02$) compared to the control subjects (6.50 ng/L vs. 5.87 ng/L vs. 5.64 ng/L, respectively). A moderate correlation between salivary BNP and plasma NT-proBNP concentrations ($p < 0.001$, $r = 0.459$) was found (395). Despite the rather small number of participants, all studies found higher natriuretic peptide levels in the saliva in HF patients compared to controls, but larger studies are needed to validate the clinical importance of the saliva values of natriuretic peptides as well as their correlation to plasma levels.

In chronic HF, inflammatory and neurohormonal activation take place. Activation of the renin-angiotensin system is responsible for the overexpression of the stress hormones, i.e., angiotensin-converting enzyme and angiotensin II. Cortisol has been implicated in the progress of chronic HF, since it binds to the mineralocorticoid receptor with an affinity equal to that of aldosterone and its

pathophysiological role may be influenced by oxidative stress (405-407), while analysis of saliva instead of serum is advantageous because salivary cortisol represents the unbound (i.e., free) hormone which is considered biologically active, while the vast majority of serum cortisol is bound to cortisol binding globulin and albumin (408). Salivary cortisol has been examined in three studies and although non-specific for HF, it should be considered, together with natriuretic peptides, the most extensively studied salivary biomarker in HF. The number of patients included in these three studies is small [229 (397), 81 (399), and 27 (402), respectively]. The LOD was 0.05 ng/mL, 0.07 ng/mL and 0.15 ng/mL respectively with values of HF patients ranging from 0.40–0.92 ng/mL, 0.19–0.55 ng/mL, and 6.88–8.33 ng/mL, respectively, in each study. Only in the third study were cortisol levels measured in controls, with values ranging from 5.43 to 6.88 ng/mL. Salivary cortisol was commonly increased in HF patients, while higher levels were associated with a reduced event-free interval and high evening levels were associated with increased mortality risk (397, 399, 402).

Galectin-3 (Gal-3) is a β-galactoside–binding lectin that plays a role in inflammatory and immune-mediated disorders. It is mainly expressed in activated macrophages and pathologically damaged cardiomyocytes and is considered as an active contributor to cardiac remodeling (including myocardial fibrogenesis) and development of HF. Gal-3 induces fibroblast proliferation and heterogeneous deposition of collagen types, eventually leading to loss of cardiac function (409, 410). Two studies have measured salivary Gal-3. In the first study, 105 HF patients (hospitalized or at routine outpatient visits) had a significantly higher cumulative risk of cardiovascular death or hospitalization when their salivary Gal-3 levels were higher than 172.58 ng/mL (393). In the second study, samples from 63 HF patients were compared to healthy controls and were significantly elevated (both in saliva and serum), with a moderate correlation ($r = 0.4$, $p < 0.01$) between serum and salivary Gal-3 levels (398).

Serum amylase is mainly found at the pancreas and salivary glands (411). In severe HF plasma amylase levels were elevated, probably because of the mesenteric venous congestion and impaired peripheral tissue perfusion (412). Two small studies measured salivary amylase in HF patients. The first (which included 33 NYHA II and

17 NYHA III patients) found decreased salivary levels of amylase in the HF population, which was attributed to the impaired secretory function of salivary glands in HF patients that led to lower content and activity of salivary amylase compared to controls (391). The second study (which included 24 NYHA I-III patients and 24 controls) found no statistically significant difference between the control and HF groups in sAA levels, although there was a strong tendency for the morning values to be higher in HF patients, especially if measured within 30 min after awakening. It should be noted that there was a strong inter- and intra-subject variation and a small number of participants, while all HF patients were on b-blocker therapy that reduces sAA levels (3). The results of these studies have put the potential value of salivary amylase or sAA as a HF biomarker into question.

HF is also associated with is hyper-lactatemia, oxidative stress, and hyperuricemia (392). In one study salivary lactate and 8-isoPGF2α from 44 patients with acute HF strongly correlated with serum NT-proBNP, while salivary uric acid did not. The LOD was 10 pg/mL and 6 μg/mL for 8-iso-PGF2α and lactate, respectively. Lactate levels positively correlated with NYHA class in non-stimulated saliva, while 8-isoprostaglandin F2α levels did not correlate to NYHA class (392). In another study, salivary 8-epiPGF2α levels were significantly higher in patients with ischemic and dilated cardiomyopathy compared to controls and patients with coronary heart disease ($p = 0.001$). 8-epiPGF2α levels negatively correlated with LVEF and positively correlated with NYHA class (401). These indices of oxidative stress may thus prove to be useful for monitoring of HF progression, while UA has also shown some ability to assess functional status, in addition to discriminating between HF patients and controls (392).

Inflammatory cytokines have been examined as markers of HF severity and prognosis in a study with 75 hospitalized HF patients (394). C-reactive protein (CRP) is a phylogenetically highly conserved plasma protein that participates in the systemic response to inflammation. Although serum and salivary levels of CRP were found to have a moderate correlation ($r = 0.594$, $p < 0.001$), salivary CRP levels were not associated with NYHA class, so it has not been associated with HF severity (394). In the same study, a weak correlation for serum–salivary IL-6 ($r = 0.288$, $p = 0.037$), and no correlations for serum–salivary IL-10 ($r = 0.068$, $p = 0.629$) or serum–salivary

BNP (r = -0.064, p = 0.628) were reported (394). Interestingly, no biomarkers in this study were associated with NYHA class and only visible oral inflammation was found to be a significant predictor of HF severity. Two major classes of cytokines have also been implicated in HF, vasoconstrictor cytokines such as endothelin and vasodepressor proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α), IL-6, and IL-1 (412, 413). These inflammatory mediators are expressed by all nucleated cell types residing in the myocardium, including the cardiac myocyte. In patients with HF, circulating as well as intra-cardiac levels of these cytokines have been reported to be elevated (414). Salivary endothelin concentrations were raised two to six-fold in chronic HF patients vs. controls (p = 0.005), with a positive correlation to respective plasma concentrations (p = 0.032) and were able to detect chronic HF with 63% sensitivity 63% and 92% specificity. Endothelin salivary levels have also been shown to reflect symptom severity by NYHA class (403). TNF-α has been identified in detectable concentrations in human saliva in patients with periodontal inflammations and oral cancers, with values that correlate well to plasma values (415, 416). This may indicate that salivary TNF-α could potentially serve as a HF biomarker if more supporting data emerged (417-421).

Mass spectrometry has also been used to analyze the saliva proteome in HF patients. In an elegant study of saliva proteomics analysis with 75 HF patients and 36 healthy controls, 728 proteins were detected by mass spectrometry; only the protein S100-A7 (S10A7) was significantly different between saliva from NYHA III/IVHF patients compared to healthy controls, but the underlying mechanisms relating S10A7 to the pathogenesis of HF are not clear and further investigations are needed to investigate the pathogenetic role of S10A7 in HF and the extent of its clinical usefulness (396).

Finally, a study dated back in 1950 had assessed the saliva concentration of sodium, chloride, and potassium in a small number of patients with congestive HF (n = 27) and compared to healthy subjects (n = 11) (404). The concept of measuring electrolytes in saliva is very interesting, especially since these are very frequently evaluated in HF patients in order to assess patients' response to treatment. Congestive HF patients had lower sodium, lower chloride, and higher potassium mean concentrations in saliva than controls, irrespectively of whether HF patients

were on a low-salt or a regular diet. Although towards the right direction, i.e., lower sodium (congestion) and higher potassium (renal failure, etc.) in HF patients, there was no relationship in electrolyte concentrations between serum and saliva (404). However, these biomarkers have been studied in one study only, and over 70 years ago, so more evidence is needed to recommend on their predictive value as HF biomarkers. Furthermore, salivary urea and creatinine concentrations, if they could be measured, would be proven to be very useful, especially for therapy monitoring in HF.

In summary, various salivary biomarkers have shown to be of value in the management of HF (**Table 3**) (3). Cortisol and Gal-3 have shown to be useful for HF prognosis. Natriuretic peptides, endothelin, Gal-3, amylase and S10A7 have shown to be useful for HF diagnosis. Finally, BNP, endothelin, cortisol, UA, 8-isoPGF2α, 8-epiPGF2α, lactate and electrolytes have been used for HF monitoring of severity progression. Regarding the correlation between saliva and serum concentrations of biomarkers, this has not been extensively studied (**Table 3**). A weak to moderate correlation was found only for endothelin (403), Gal-3 (398), CRP and IL-6 (394), while no correlation was found for NT-proBNP (400), BNP and IL-10 (394).

Table 3. Saliva biomarkers that have shown a potential role in HF management (3).

Author [Reference]	Year	Saliva Biomarker	Range (in Total Study Population)	Correlation between Saliva and Serum Levels	Clinical Usefulness
Foo et al (400).	2012	NT-proBNP	18.3–748.7 pg/mL	No correlation	Diagnosis
Joharimoghadam et al (395).	2017	BNP	4.96–6.85 ng/L	Not assessed	Diagnosis Monitoring
Denver et al (403).	2000	Endothelin	0.50–23.56 fmol/mL	r = 0.536, p = 0.032	Diagnosis Monitoring
Zhang et al (398).	2016	Gal-3	2.8–2510 ng/mL	r = 0.4, p < 0.01	Diagnosis

Zhong et al (410).	2019	Gal-3	16.04–869.3 ng/mL	Not assessed	Prognosis (association with cardiovascular death or hospitalization)
Zhang et al (396).	2017	S10A7	Not assessed	Not assessed	Diagnosis
Hammer et al (397).	2016	Cortisol	0.08–1.28 ng/mL	Not assessed	Prognosis (association with mortality)
Alhurani et al (399).	2014	Cortisol	0.09–0.55 µg/dL	Not assessed	Prognosis (association with cardiac event free-survival)
Jekell et al (402).	2004	Cortisol	15–23 nmol/L	Not assessed	Monitoring
Klimiuk et al (391).	2020	Uric Acid	0.5818–0.862 ng/mL	Not assessed	Diagnosis, Monitoring
Ghimenti et al (392).	2020	8-isoPGF$_{2\alpha}$	25–60 pg/mL	Not assessed	Monitoring
Wolfram et al (401).	2005	8-epiPGF2α	43–111 pg/mL	Not assessed	Diagnosis, Monitoring
Ghimenti et al (392).	2020	Lactate	190–3790 mmol/L	Not assessed	Monitoring
Klimiuk et al (391).	2020	Amylase	0.12–0.21 µmol/mg	Not assessed	Diagnosis (lower levels in HF)
White et al (404).	1950	Sodium, Chloride, Potassium	7.4–27.9 12.8–30.8 16.2–29.7 mEq/L	No correlation found	Monitoring

BIOMARKERS IN OTHER FLUIDS

Urinary biomarkers

Urine is also a biological fluid that can be easily collected with a non-interventional procedure and even in a primary health care setting. Beginning with the gold standard, urinary NT-proBNP levels show good stability in a 24-month follow-up of patients with stable HF and may be a useful tool for monitoring patients (422). A study of subjects suffering from HF but without official diagnosis (n=1.308) found that both urinary and plasma NT-proBNP had high predictive values for HF diagnosis, but urine was more specific than plasma (67% vs 41%) and also less costly (423).

Renal dysfunction is a prevalent and independent predictor of adverse outcomes in patients with acute decompensated HF (424), and should always be investigated as a potential pathway in this multifactorial disease. Urinary biomarkers may be able to identify renal injury more quickly and specifically than serum creatinine which may take up to 72 hours to rise, and is generally unable to differentiate pre-renal causes from an acute spiramatic injury. Furthermore, creatinine production is directly related to muscle mass which may be redused in elderly or cachectic patients (425). Towards this direction several urinary biomarkers of tubular injury have been investigated as potential prognostic biomarkers of HF.

Albumin is the most abundant protein in serum. Urinary albumin excretion results from the disruption of the glomerular basement membrane. It is usually correlated with diabetic nephropathy and hypertension, but also with CVD. In a study with HF patients (n=5.010, NYHA II-IV), albuminuria was correlated to elevated risk of all-cause mortality and HF hospitalization (426-428). Meta- analysis of these three large studies suggest that albuminuria in patients with HF is a marker for significant morbidity and and all-cause mortality (425).

In a large analysis of four prospective, observational cohorts with theoretically healthy subjects (n=22.756), urinary albumin creatinine ratio (UACR)

was associated with increased risk both for HFpEF [(HR), 1.33; 95% CI, 1.20-1.48; p<0.001 and HFrEF development (HR, 1.21; 95% CI, 1.11-1.32; p<0.001) (429).

Another study with 24.433 participants and a median follow-up of 9.2 years reported higher risk of incident HF with increasing UACR categories (10 to 29 mg/g [HR]: 1.49; 95% [CI]: 1.26 to 1.78; 30 to 300 mg/g HR: 2.32; 95% CI: 1.93 to 2.78; >300 mg/g HR: 4.42; 95% CI: 3.36 to 5.83) in the fully adjusted model (430). Furthermore, association between UACR and HFpEF development was greater than with HFrEF (HR comparing highest vs. lowest ACR category: 6.20; 95% CI: 4.15 to 9.26 vs. HR: 4.37; 95% CI: 3.00 to 6.25, respectively; p = 0.05) (430).

Urinary neutrophil gelatinase-associated lipocalin (U-NGAL) on the first day of admission was found to be an early predictor of acute kidney injury (AKI), defined as a 25% decrease in eGFR, which in turn is a strong prognostic factor for long-term outcomes in patients with acute decompensated HF (431). In a small study with 260 HF patients the high-U-NGAL group had a significantly higher occurrence of AKI during hospitalization than the low-U-NGAL group (*P*=0.0012) and exhibited a worse prognosis in all-cause death (hazard ratio 2.07; 95%CI 1.38-3.12, *P*=0.0004), cardiovascular death (hazard ratio 2.29; 95%CI 1.28-4.24, p=0.0052), and HF admission (hazard ratio 1.77; 95%CI 1.13-2.77, p=0.0119) (432). In a second study, urine NGAL level in decompensated HF patients (n=100) was a good marker for predicting AKI (at 12 ng/mL cut-off value), however did not predict the diuretic dose requirement (433). In a larger study (n=547) with stable NYHA II HF patients of various causes (ischemic, dilated, other) NT-proBNP (P= 0.001) and NGAL (P = 0.004) were significant predictors of adverse cardiac events and their combination was even stronger predictor of the primary endpoint than NGAL or NT-proBNP alone (434). At a prospective study (GALLANT, n=186, 61% males) NGAL predicted re-hospitalization for AHF and all-cause mortality in 30 days (P= 0.001) while BNP approached significance (P= 0.052). On the contrary, neither serum creatinine nor e-GFR was significant. The addition of discharge NGAL over BNP alone improved classification by 10.3% in those with events and 19.5% in those without events (P= 0.01). Subjects with both BNP and NGAL elevated were at significant risk [hazard ratio (HR) = 16.85, P= 0.006], as were subjects with low BNP and high NGAL (HR = 9.95, P=0.036) (435). An even larger study than the previous mentioned so far

(n=927) with acutely decompensated HF patients) showed that uNGAL predicts a worsening renal function (defined as an increase in creatinine of 0.5 mg/dL, an increase of more than 50% above first value or initiation of renal replacement therapy, within the first 5 days) but not better compared to the first creatinine value (436). Furthermore, for the same population serum NGAL outperformed urine NGAL but neither was superior to admission or peak serum creatinine for predicting adverse events (437). On the other hand, in a third study with also small sample size (n=83) urinary NGAL, was and poorly correlated with the estimated glomerular filtration rate (eGFR; Spearman ρ ≤0.28) and did not predict AKI (438). In this study, urinary IL-18 was significantly associated with all-cause mortality (hazard ratio 1.48, 95% confidence interval 1.16-1.87; P = .001) in patients with HF (438). These conflicting conclusions show that uNGAL may predict a worsening renal function in acute HF patients, may have prognostic value for adverse events but not necessarily better than serum creatinine. Therefore, more data are needed for its role as a useful biomarker in HF.

Kidney Injury Molecule 1 (KIM-1) is a trans-membrane glycoprotein with a small intracellular part and a larger extracellular part. Within 24 to 48 hours after kidney injury, KIM-1 is increased in proximal tubular epithelial cells. Similar to NGAL, there are some studies of KIM-1 in patients with HF. Indicatively in a study evaluating the usefulness of urine KIM-1 and NGAL for the detection of AKI in a group of ICU patients with HF who underwent coronary angiography both biomarkers were useful but NGAL showed superior sensitivity and specificity (439). In a study with stable HF patients NYHA II to IV (n=90), the levels of KIM-1 were significantly higher than controls and were associated with serum NT-proBNP levels, without any significant difference whether they had CKD or not (440). In the same study, urinary N-Acetyl-β-D-Glucosaminidase (NAG) was elevated in association with NT-proBNP and eGFR, but not with blood pressure, hemoglobin, or LVEF in NYHA II to IV HF patients and it was predictive of the combined outcomes of death, heart failure hospitalization, and heart transplantation (440). A second study with the same two urinary biomarkers (i.e. KIM-1 and NAG) and 150 HF patients found significantly elevated KIM-1 values in the HF arm compared to controls (1100, IQR 620-1920 vs. 550, IQR 320-740 ng/g

urinary creatinine, P < 0.001), significant correlation with LVEF (r = -0.37, p< 0.001) and NYHA class (r = 0.5, p<0.001), while NAG values showed a weaker response (6-22 U/gr creatinine) but correlated significantly with LVEF (r = -0.18, P= 0.015) and clinical status (r = 0.22, p= 0.04). KIM-1 and NAG were also predictors of all-cause mortality and the composite of all-cause mortality and re-hospitalization for heart failure (all P<0.05) (441). Further research is needed to clarify the role of urinary KIM-1 and NAG in the diagnosis and management of cardiorenal syndromes and HF stratification.

Another urinary biomarker is C-type natriuretic peptide (CNP) which is produced in the kidney and the endothelium. As part of the natriuretic peptide family it may provide great information in terms of renal impairment and the cardio-renal interaction in acute HF. This hypothesis was tested in a small study with 58 patients with acute HF (442). Apart from urine and plasma CNP (mainly it's active mature form CNP22 and its inactive form NT-CNP53) they also studied urinary KIM-1 and NGAL, and plasma NT-proBNP and compared them with 20 healthy control subjects without known cardiovascular disease (CVD). Median urinary excretion of CNP and KIM-1 was significantly higher in HF patients than controls, while U-NGAL excretion was unchanged (p=0.94). There was no significant relationship between any urinary biomarker and NYHA class or LVEF. Plasma CNP22 was elevated in HF compared to controls but without statistically important association to urine levels (p=0.28). Plasma NT-CNP53 was unchanged without any relationship to urinary excretion. Cox regression analysis revealed that urinary NT-CNP53 excretion was significantly predictive of mortality and all-cause rehospitalization/death, even after adjusting for age, urinary protein/creatinine ratio, and plasma NT-proBNP [mortality rate (95%CI): 73.1% (57.9-92.3) vs. 96.7% (90.5-100); rehospitalization/death rate (95%CI): 46.3% (30.6-70.0) vs. 69.8% (55.1-88.5)] (442). These data support a potential role for NT-CNP53 for risk stratification in HF patients, while urinary KIM-1 and NGAL demonstrated no significant predictive value.

A urine biomarker with utmost clinical importance is sodium. High spot urine sodium after diuretic administration was significantly associated with urine output in

a model adjusted for age, renal function, and blood urea nitrogen (p =0.02) (443). Higher urine sodium was associated with lower risk of hospitalization or emergency visit within 30 days (odds ratio [OR] 0.82 [95% confidence interval 0.72-0.94] per 10mmol/L increase (p < 0.001), while spot urine sodium greater than 65mmol/L and urine output greater than 1200mL identified a lower-risk group for outpatient management (441). In another study the daily urinary sodium excretion from 2961 participants, after adjustment for potential confounders, was not associated with hs-cTnT, hs-cTnI, and NT-proBNP concentrations (444). In contrast, a lower daily potassium excretion was nonlinearly associated with higher hs-cTnT and NT-proBNP. Associations were similar after further adjustment for estimated glomerular filtration rate, albuminuria, blood pressure, and serum potassium, thus making urinary potassium a potential biomarker for cardiac dysfunction and/or cardiomyocyte injury diagnosis.

Global proteomic analyses of urine samples using techniques such as 2D-DIGE and mass spectrometry have produced panels of biomarkers that could be useful in detecting HF. In a study with 197 HF patients twenty proteins of interest were identified and validated by western blot; after analysis alpha-1-acid glycoprotein 1 (ORM1), a transport protein in the bloodstream which has a regulatory dampening effect on the inflammatory cascade was able to diagnose HF with 91% sensitivity and 85% specificity (445). Urinary proteomic analysis by means of capillary electrophoresis coupled to mass spectrometry (CE-MS) has identified a panel of urinary peptides, termed HF1, which diagnoses hypertensive subjects with diastolic dysfunction from controls (446). A study with 829 subjects (622 HF, 207 controls) found that HF1 discriminates HF (either HFrEF or HFpEF) from healthy controls and subjects with CHD (447). The AUC using HF1 for HF diagnosis was 0.94 (95% CI, 0.92–0.96), compared to the AUC for BNP which was 0.98 (95% CI, 0.97–0.99) (447). Adding HF1 to BNP increased the AUC to 0.99 (0.98–0.99), p<0.001, and led to a net reclassification improvement of 0.67 (95% CI, 0.54–0.77), p<0.001 (447). However, HF1 less prognostic utility than BNP for all-cause mortality and did not add prognostic information when combined with BNP (447).

Quantitative real-time polymerase chain reaction has been used for the expression of microRNA-423-5p in urine (and serum) samples of acute HF patients. Both urinary and serum microRNA-423-5p had relatively high predictive performance for AKI onset and predicted a poor 180-day survival, while this predictive value was even more significant when combined with urinary NGAL (448).

In a small study comparing acute ischemic HF patients (n=30) with stable chronic HF patients (n=30) and controls (n=30), urine calcium integrin binding protein-1 (CIB1) was positively correlated ($p < 0.05$) to serum NT-proBNP in the AHF arm (449). CIB1 was significantly higher in AHF patients than in stable chronic HF patients, while in the chronic HF arm the diagnostic value of urine CIB1 outperformed that of serum NT-proBNP with a diagnostic accuracy of 0.854, sensitivity 86.6% and specificity 82.5%, respectively. The diagnostic accuracy of NT-proBNP was 0.823, the sensitivity was 83.5% and the specificity was 85.9%, respectively. The levels of urine CIB1 and serum NT-proBNP were significantly lower in the control group ($p < 0.05$) (449).

Cortisol is a biomarker that has been correlated with HF through inflammatory and neurohormonal activation that take place as a response to the failing heart. Cortisol has been implicated in the progress of chronic HF, since it binds to the mineralocorticoid receptor with an affinity equal to that of aldosterone and its pathophysiological role may be influenced by oxidative stress (3). Cortisol is found in similarly high concentrations in blood and urine (450) ranging from 10-100ng/mL in healthy individuals and raised in HF patients. Vasodepressor proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α and Interleukin-6 (IL-6) have also been implicated in HF prognosis (all cause death and HF hospital admissions in HFpEF) (172) and can also be detected in urine with ranges from 0.03-0.4ng/mL and 20-30pg/mL respectively, but their role in HF diagnosis needs further investigation (450).

Cortisol and IL-6 have also been detected in sweat in concentrations of 8-142pg/mL and 7-16pg/mL in the same study (450) but their role in HF diagnosis also needs further investigation.

In summary, urinary biomarkers have shown to be of value in HF prognosis (Table 4). NGAL, KIM-1 and NAG are the most studied biomarkers, and have been found to correlate with HF prognosis. NT-proBNP has also been detected in urine and can be used for HF diagnosis. Regarding the correlation between urinary and serum concentrations of biomarkers, this has not been extensively studied (Table 4). Further research is required to establish urine biomarkers as useful tools for HF management.

Table 4. Urine biomarkers that have shown a potential role in HF management

Author [Reference]	Year	Urinary Biomarker	Range (in Total Study Population)	Correlation between Urine and Serum Levels	Clinical Usefulness
Ng et al (423).	2005	NT-proBNP	0.54-1.103.8 fmol/mL	Not mentioned	Diagnosis
Cortes et al (422).	2007	NT-proBNP	60-120pg/mL	1:9-1:15	Monitoring
Comnick and Ishani (425).	2011	Albumin	N/A	N/A	Prognosis
DeBoer et al (429).	2018	Albumin Creatinine Ratio	N/A	N/A	Prognosis
Maisel et al (435).	2011	NGAL	60-200ng/mL	N/A	Prognosis
Nakada et al (432).	2017	NGAL	N/A	N/A	Prognosis
Labr et al (434).	2018	NGAL	N/A	N/A	Prognosis
Verbrugge et al (438).	2013	IL-18	10.97-46.56 pg/mL	N/A	Prognosis (AKI in

					ADHF)
Damman et al (440).	2010	KIM-1	188-537) ng/g creatinine	N/A	Prognosis
Damman et al (440).	2010	NAG	6–17 U/g creatinine	N/A	Prognosis
Jungbauer et al (441).	2011	KIM-1	620-1920 ng/g creatinine	N/A	Prognosis, Monitoring
Jungbauer et al (441).	2011	NAG	6-22 U/g creatinine	N/A	Prognosis
Zakeri et al (442).	2013	CNP22,CNP53	N/A	CNP22 limited association (p=0.28, p=0.04) CNP53 no relationship	Prognosis
Brinkley et al (443).	2018	Sodium	134-141 mmol/L	N/A	Prognosis (30 day)
Hou et al (445).	2014	ORM1	1.033.02-13.244,76 ng/mg	N/A	Diagnosis
Zhang et al (448).	2021	microRNA-423-5p	N/A	N/A	Prognosis (180 day)
Zhang et al (449).	2017	CIB1	0.65-8.23 µg/L	N/A	Diagnosis
Steckl (450).	2018	Cortisol	0.03-0.3 µM	N/A	Diagnosis
Campbell et al (447).	2020	Proteomic classifier HF1	0.30-2.46	N/A	Diagnosis

BIOMARKER-GUIDED MANAGEMENT OF HF

So far many biomarkers have been identified, mainly in plasma/serum but also in other biological fluids like saliva and urine, that may have an important role in diagnosis, prognosis and risk stratification of HF. The important question and what could launch their application is whether they can prevent HF and whether they can assist or even better guide HF management.

a) Biomarkers for the Prevention of HF development

A pooled cohort analysis from three large studies (n=12.987, mean age 55 years; 55% women) investigated whether the two most studied biomarkers of chronic myocardial injury (hs-TnT ≥6 ng/L) and stress (NT-proBNP ≥100 pg/mL) can guide CV risk stratification and treatment monitoring among adults with elevated BP that do not meet the criteria for anti-hypertensive treatment, a very common predisposing factor of HF, according to 2017 AHA guidelines (451). Over a 10-year follow-up, subjects with elevated BP or hypertension not recommended for antihypertensive treatment with versus without one elevated biomarker had a 10-year CV incidence rate of 11.0% and 4.6% respectively (449). This means that elevated biomarkers at baseline could identify individuals not currently recommended for antihypertensive medication that are however at high risk for CV events and thus leading to more intensive BP control (451).

The Cardio-Oncology Study Group of the Heart Failure Association and the Cardio-Oncology Council of the European Society of Cardiology published a position statement conserning the role of serum biomarkers in cancer patients receiving cardiotoxic cancer therapies (452). Baseline measurements of NPs and cTnT/I should be considered to identify potential haemodynamic stress and cardiomyocyte injury related to underlying cardiac disease pre-treatment, especially when CVD is present (452). Although an increase in a cardiac biomarker will rarely justify a stop of evidence-based cancer therapy, serial biomarker kinetics should be acquired during and after chemotherapy, in order to guide the decisions to increase monitoring frequency, initiate cardioprotective treatment (e.g. ACEI, HER2-targeted therapies), or justify further cardiac investigations (452).

b) Application of Biomarkers in clinical trials in HF

The role of biomarkers in RCTs is ever expanding. Biomarkers have been included in the enrollment criteria in order to verify that enrolled subjects have HF, to stratify patients and to evaluate differential efficacy or safety (119). Assays for NPs are being increasingly used in RCTs for both acute and chronic HF to confirm a clinical diagnosis of HF requiring levels above a diagnostic cut point for patients to be enrolled. BNP and NT-proBNP are the biomarkers mostly included in clinical trials **(Table 5)**. Another biomarker that has been used for HF diagnosis is midregional proANP, in the BACH trial, at a threshold of 120pmol/L and it was non-inferior to a BNP level of 100pg/mL for the diagnosis of AHF (466).

Biomarkers may also provide information about the mechanisms of action by which changes in clinical condition may be explained. In the PARADIGM-HF trial, which compared the ARNI LCZ696 (200 mg b.i.d.) with the ACEI enalapril (10 mg b.i.d.) in 8399 patients with HFrEF, biomarker kinetics provided useful insights into the effect of treatment on the progression of HF. The ARNI led to an early peak in BNP and a reduction in NT-proBNP and cTnT compared to ACEI (467). The kinetics of biomarkers paralleled the clinical status of subjects (disease progression, dose intensification [p=0.003] or ED visits for worsening HF [p=0.001]), with these events being evident within 30 days after randomization, reflecting the change in biomarker levels (452). Since troponin reflects myocardial injury (possibly related to increased wall stress) these findings suggest that this may account for the more effective prevention of the clinical progression of HF by ARNI than enalapril (467). Serial measurements of NT-proBNP, hs-CRP, or cTnT in the HF-ACTION study (Heart Failure and a Controlled Trial Investigating Outcomes of Exercise Training) revealed that plasma levels of biomarkers did not significantly improve at 3 months with a structured exercise training program, and that more intensive exercise also did not associate with a reduction in serial levels (468). These findings challenge the hypothesis that exercise training improves biomarkers in CHF patients (468).

By enlighting the pathohysiologic mechanisms of HF, another use of biomarkers in clinical trials may be the identification of high-risk patients of clinical events, which is necessary in order to better ensure sufficient powering of the trial.

Table 5.Clinical trials using natriuretic peptides for biomarker-guided therapy							
Study	**N**	**Population**	**Timepoints**	**Primary end point**	**Follow up duration (median)**	**Improved Outcome**	**Ref**
Troughton et al, 2000	69	NYHA II-IV, HFrEF	Every 3 months	Composite of CVD, HFH, HF decompensation	9.5 months	Yes	(453)
STARS-BNP, 2007	220	NYHA II-III, LVEF<45%, on OMT	Every month for 3 months, then every 3 months	CVD or HFH	15 months	Yes	(454)
TIME-CHF, 2009	499	NYHA II-IV, LVEF≤45%	1,3,6,12,18 months after discharge	18-month survival free of hospitalizations and QoL	18 months	No	(455)
Anguita et al, 2010	60	NYHA III-IV,	NA	Composite of all-cause mortality and HFH	16±4 months	No	(456)
PRIMA, 2010	345	AHF	Discharge, 2weeks,1,3,6,9,12, 15,18,21,24 months	Survival without HFH	24 months	No, but advanced detection of HF events	(457)
SIGNAL-HF, 2010	252	NYHA II-IV, LVEF<50%	Baseline, <10days, 1,3,6,9 months	Composite of days alive, days out of hospital, and KCCQ score	9 months	No	(458)
BATTLESCARRED, 2010	364	AHF	Every 3 months	All-cause mortality	36 months	No, but improves long-term mortality in ≤75years old	(459)
Berger et al, 2010	278	NYHA III-IV, HFrEF, AHF	1,3,6,12 months after discharge	All-cause mortality, HFH	12 months	Yes	(460)
STARBRITE, 2011	130	NYHA III-IV, LVEF≤35%, AHF	1 week, 1,2,3,4 months after discharge	Survival without HFH	3 months	No	(461)
UPSTEP, 2011	279	NYHA III-IV,	Week 2,6,10,16,	Composite of all-cause death,	12 months	No	(462)

		LVEF<40%, AHF in past month	24,36,48, then every 6 months	hospitalization and diuretic up-titration			
PROTECT, 2010	151	NYHA II-IV, LVEF≤40%, AHF event	Every 3 months	Composite of worsening HF, HFH, clinically significant VA, ACS, cerebral ischemia, and CVD	6 months	Yes	(463)
NORTHSTAR, 2013	407	NYHA I-II (85%), LVEF≤45%	Every 1-3months	Composite of all-cause mortality and HFH	30 months	No	(464)
HABIT, 2013	167	NYHA I-IV, AHF, 70% HFrEF 30% HFpEF	Every day	ADHF events: CV death, ADHF admission or iv therapy or changes in oral HF medications	60 days after discharge	Yes	(100)
GUIDE-IT, 2017	110	LVEF<40%, high risk patients for HF events	At 2 weeks and every 3 months	Composite of CVD, HFH	15 months	No	(104)
TIME-CHF, 2017	499	NYHA II-IV, LVEF≤45%	1,3,6,12,18 months after discharge	Recurrent HFH and all-cause hospitalizations and/or all-cause death	5 years follow-up	Yes only for <75 years; improved HF hospitalization-free survival	(105)
PRIMA II, 2018	405	ADHF with NT-proBNP >1700 ng/L	Not predefined	Composite of all-cause mortality and HFH in 180 days, and number of days alive out of the hospital in 180 days	6 months	No; in biomarker arm discharge NT-proBNP more reduced	(465)

ACS: acute coronary syndrome, ADHF: acutely decompensated heart failure, CVD: cardiovascular death, HFH: heart failure hospitalization, KCCQ: Kansas City Cardiomyopathy Questionnaire, NA: Not applicable, OMT: optimal medical treatment, QoL: quality of life, VA: ventricular arrhythmia.

Again, for instance, in the PARADIGM-HF trial patients were included if their palsma BNP was higher than 150 pg/mL or their NT-proBNP higher than 600 pg/mL (467).

c) Biomarkers in trials with positive results for HF management

The first study to investigate the effectiveness of biomarker-guided management versus standardized clinical assessment was published 22 years ago and measured BNP in HFrEF patients, functionally NYHA II class (n=69) (469). During a median 9month follow-up there were fewer total CV events (death, HF hospitalization, or HF decompensation) in the BNP group than in the clinical group (p=0.02) and the first event took place later (p=0.034) (469). This innovative study paved the path for subsequent prospective studies which however had conflicting results (470).

The most positive results showed that NP-guided treatment reduced both mortality and HF admissions. A systematic review and meta-analysis of 19 trials (n=4.554) with average ages 62-80 years, investigated whether NP-guided treatment of HF reduces all-cause mortality and HF admissions compared to clinical assessment alone (471). Pooled risk ratio estimates for all-cause mortality (16 trials, 4.063 participants) were 0.87, 95% CI 0.77 to 0.99 and for HF admissions (11 trials, 2822 participants) were 0.80, 95% CI 0.72 to 0.89 (471). These data suggest that NP-guided treatment is beneficial in reducing HF admissions and all-cause mortality (471). Another meta-analysis of nine RCTs and 1.914 subjects reported that NP-guided therapy significantly reduced overall mortality and HF-related hospital admissions, without significant results in HF-related mortality and overall hospital admissions (472).

There are meta-analyses with positive but more modest results. A large meta-analysis of 14 studies (n=3.004) concluded that BNP-guided treatment significantly decreased the risk of HF hospitalization (RR 0.79, 95 % CI 0.63-0.98, p=0.03) compared to clinical group, but did not significantly alter the risk of all-cause mortality (RR 0.94, 95 % CI 0.81-1.08, p=0.39) or all-cause hospitalization (RR 0.97,

95 % CI 0.89-1.07, p=0.56) (473). Nevertheless, the BNP changes seemed to be a significant modifier and only when a substantial reduction of BNP levels can be achieved, can BNP-guided therapy improve the clinical outcomes of CHF patients (460). The same results came from another systematic review of two RCTs (n=1.674), with subjects without HF but with at least one risk factor for CVD (461). The mean age ranged between 64.1 and 67.8 years and the follow-up from 2 to 4.3 years (474). NP-guided treatment reduced the risk of HF hospitalisation (RR 0.52, 95% CI 0.40 to 0.68; 2 studies; 1.674 participants; moderate-quality evidence), but there was uncertainty concerning the effect of NP-guided treatment on all-cause mortality (RR 0.90, 95% CI 0.60 to 1.35; 2 studies; 1.354 participants; low-quality evidence) (474).

According to an insilico study using data from BIOSTAT-CHF (BIOlogy Study to Tailored Treatment in Chronic Heart Failure) trial, in which all patients were divided in 3 theoretical treatment scenarios, i.e. patients up-titrated to >50% of recommended doses; patients up-titrated according to a biomarker-based treatment selection model, or no up-titration to >50% of recommended doses, multivariable Cox regression estimated that if all patients were up-titrated successfully, an estimated 9.8, 1.3, and 12.3 events per 100 treated patients could be prevented at 24 months by ACE inhibitor/ARB, beta-blocker, and MRA therapy, while for the second scenario the percentages would be 9.9, 4.7, and 13.1 respectively and the biomarker based up-titration might have resulted in fewer deaths or hospitalizations (475).

Three promissing studies concerning Ca-125 guided treatment versus standard treatment were recently published. The CHANCE-HF trial evaluated the prognostic effect of Ca-125-guided therapy versus standard of care after AHF hospitalization and showed that after discharge, bio marker-guided therapy that keeps its values below 35 U/mL is associated with a reduced risk of adverse 1-year outcomes, mainly due to significant reduction of the rate of rehospitalization (476). Similarly, the IMPROVE-HF RCT compared Ca-125-guided diuretic treatment vs. usual care in patients with AHF and renal dysfunction found that biomarker-guided intravenous diuretic therapy resulted in better renal performance at 3 and 30 days and decreased the risk of adverse clinical endpoints at 30 days (477). Finally,

as a marker of extravascular congestion, Ca-125 kinetics could guide diuretic treatment on a more chronic base (478).

STADE-HF was a blinded prospective RCT that investigated whether pre-discharge sST2 levels of AHF hospitalized patients (n=123) could serve as a guide for medical management in order to reduce HF related readmissions (479). Usual treatment (without sST2) was compared to sST2 treatment group, in which sST2 level was known and used on Day 4 of hospitalization to guide the treatment, and the primary endpoint was all-cause readmission rate at 1 month (466). Although the sST2-guided therapy over a short period of time did not reduce readmissions (p=0.11), sST2 was significantly associated with duration of hospitalization, and the decrease in sST2 value was associated with decreased rehospitalizations (479).

A post hoc analysis of the TIME-CHF study (n=499, LVEF ≤ 45%, NYHA class II-IV) with a 19-month follow up showed that diuretic up-titration was beneficial for patients with high interleukin-6 (IL6) or high hsCRP (interaction, *P* = 0.013 and p= 0.001), it was associated with poor outcome in patients with high blood urea nitrogen or prealbumin (interaction, p=0.006 and p=0.001) and spironolactone up-titration was associated with lower risk of HF hospitalization or death in patients with high CysC (interaction, p=0.021) (480). These data suggest that biomarker use for decision-making may be very promising (480).

Finally, MR-proADM concentrations above 44.6 pg/mL in hospitalized patients with AHF (n=764) could identify those with disproportional benefit if treated with diuretics (interaction p values < 0.001), since they had increased risk of death ([HR] 1.87, 95% [CI] 1.57-2.24; p<0.001) (481).

d) Biomarkers in trials with neutral or negative results for HF management

The recent GUIDE-IT trial, which is the largest NP-guided trial in HF, was neutral for the biomarker-guided arm compared to optimal guideline-directed medical therapy (GDMT) (482). High risk HFrRF patients (n=894) with a median follow-up of 15 months had similar risk (adjusted [HR] 0.98; 95% [CI], 0.79-1.22;

p=0.88) for the composite primary end point (time-to-first HF hospitalization or CV death) and CV mortality ([HR] 0.94; 95% [CI] 0.65-1.37; p=0.75), while the secondary end points (all-cause mortality, total HF hospitalizations, days alive but not hospitalized for CV reasons, the individual components on the primary end point, and adverse events) did not differ significantly (482). A secondary analysis of this RCT revealed that at six months only a 15.5% of HF patients had been up-titrated to an optimal GDMT, however that happened in both arms of the trial (483). According to these results, although there were opportunities to titrate medications, GUIDE-IT failed to achieve treatment benefit because of therapeutic inertia in clinical practice, or unrealistic GDMT targets (483). One objection could be the fact that the study design in GUIDE-IT offered more than what is usually reffered as usual care, since it was delivered in tertiary care centers by experienced HF specialists, with almost monthly visits in a single year (104). Furthermore, another reason could be the fact that a large proportion of patients in NP-guided trials have been included in an early phase after decompensation (484).

Another trial, PRIMA II showed that in AHF hospitalized patients NT-proBNP-guided therapy using a pre-defined target of more than 30% reduction did not reduce readmission or mortality at 180 days ([HR] 0.96; 95% [CI] 0.72–1.37; p=0.99), the median number of days alive outside of the hospital (p=0.39), or any of the secondary end points, compared to conventional therapy (485). On top of that, another study showed that in AHF hospitalized patients, enhanced therapy based on pre-discharge plasma levels of NT-proBNP was not associated with better long-term outcomes (486).

A second study showed that these two biomarkers identified patients with DM type 2 at elevated HF and cardiac event risk and predict which patients may benefit most from dapagliflozin (487). Dapagliflozin reduced the risk of the CV death or HF hospitalization in patients with DM type 2 in DECLARE-TIMI 58, a double-blind RCT (n=14.565, median follow-up 4.2 years) (488). Those patients were stratified by baseline biomarker levels and those with higher levels of NT-proBNP and hsTnT had higher event rates (Q4 vs Q1: NT-proBNP: 13.7% vs 1.0%; hsTnT: 11.8% vs 1.4%; P-trend <0.001) (487).

A study using procalcitonin (PCT) values (> 0.2 μg/L) to initiate early antibiotic treatment in patients presenting with symptoms and signs of AHF and elevated NPs, found that the strategy of PCT-guided initiation of antibiotics was not more effective than a standard care strategy in improving 90-day all-cause mortality (P = 0.316), 30-day all-cause mortality and HF readmission, and rate of pneumonia (489).

e) Biomarkers as Therapeutics for HF

In addition to serving as biomarkers, NPs may serve as therapeutics for both HFrEF and HFpEF (490). NPs could be increased either by preventing degradation by neutral endopeptidases such as in the OVERTURE trial (Omapatrilat Versus Enalapril Randomized Trial of Utility in Reducing Events), the PARAMOUNT (Prospective Comparison of Angiotensin Receptor Neprilysin Inhibitor (ARNI) With Angiotensin II Receptor Blocker (ARB) on Management of Heart Failure With Preserved Ejection Fraction) and PARADIGM-HF (Prospective Comparison of ARNI With Angiotensin-Converting Enzyme Inhibitor (ACEI) to Determine Impact on Global Mortality and Morbidity in Heart Failure) studies or by the exogenous administration of BNP (377, 467, 491). In PARADIGM-HF study patients with lower NT-proBNP values (≤1000 pg/mL) had reduced rates of CV death or HF hospitalizations compared with those who did not (454). In PARAMOUNT study valsartan/sacubitril significantly reduced NT-proBNP by ≈15% compared with valsartan, while at 36 weeks valsartan/sacubitril significantly reduced left atrial volume by ≈5% and improved NYHA class compared to valsartan (377). The results of PARAGON-HF [Prospective Comparison of ARNI With ARB Global Outcomes in Heart Failure With Preserved Ejection Fraction] were recently released and showed that Among patients with HFpEF, sacubitril/valsartan was not effective at reducing the incidence of CV death or HF hospitalization compared to valsartan (492).

f) Further Biomarker Implication in Clinical Practice

As presented so far, the majority of studies of several HF biomarkers have focused on their prognostic value, either in risk stratification of patients already diagnosed with HF, or in appearingly healthy subjects at higher risk for HF onset. With the exception of NPs, fewer studies of other biomarkers have focused on

diagnosis of HF. BNP and NT-proBNP are considered to be the gold standard biomarkers for the diagnosis of HF and are proposed by the current European and American guidelines; nonetheless, efforts to develop other biomarkers or panels with combinations of them, in order to improve the diagnostic specificity or diagnose specific causes of HF (e.g. cardiomyopathies such as amyloidosis) in an evidence-based, cost-effective way, would be more than welcome. Finally, even fewer studies have focused on therapy monitoring, although this could potentially be the most useful application of biomarkers.

Despite a rapid evolution in biomarker science, there are still many difficulties to overcome before their widespread use. Such difficulties are logistics, regulatory barriers, and challenges in optimal evaluation and clinical use of biomarkers (490). Furthermore, more standardized methods for analyses, larger populations in clinical studies, more representative study samples, rigorous statistical assessment methods should be applied in order to contextualize findings and have exponential gains in clinical information obtained (492), in order to provide an answer to the most important question, i.e. whether a biomarker can effectively lead to further diagnostic testing, modification of therapeutics, and cost-effective improvement of patients' outcome. Until then, there will still be a suboptimal use of biomarkers in decision-making, in the allocation of HF medical therapies or more advanced therapies such as heart transplantation or LVAD implantation (492).

SECOND PART

RESEARCH

1. OBJECTIVES OF THE STUDY

In our study we evaluated the role of salivary biomarkers in the diagnosis and risk stratification of HF; the salivary concentration of NT-proBNP, which is an established HF biomarker in serum, and the salivary value of cortisol, a biomarker that has mainly been used for other diseases other than HF.

The primary aim of the study was to investigate whether salivary NT-proBNP values differ between patients with HF (acute or chronic) compared to other patients who have dyspnea and exercise intolerance but are not diagnosed with HF such as obese and hypertensive patients, in which case the biomarker in question could be used for the diagnosis of HF. We also investigated whether salivary NT-proBNP values differ between patients with acute HF and patients with chronic HF, indicating a potential value of the biomarker in risk stratification of HF.

An important aim of the study was to evaluate the correlation between salivary and serum NT-proBNP values in patients with acute HF (acute de novo or acute decompensated) and chronic stable HF as well as non-HF patients who served as controls (obese and hypertensive).

We also studied potential associations of the salivary NT-proBNP levels with any clinical, laboratory or imaging parameters that either are affected in HF (e.g. the 6MWD, NYHA functional class, left ventricular internal diameter, left ventricular filling pressures, kidney function, electrolytes etc) or are known to affect the levels of serum NT-proBNP (e.g. weight, BMI, presence of atrial fibrillation etc).

The assessment of the response to treatment was also evaluated in patients who were admitted to the hospital with acute HF and treated; the evolution of the biomarker values from admission to discharge and at 4 weeks after discharge was studied. On the other hand, the repeatability of the biomarker was assessed by comparing its levels in stable HF and non-HF patients between two time points, 4 weeks apart.

Regarding cortisol, similar to our studies of NT-proBNP, we investigated whether salivary cortisol may be used for HF diagnosis (distinguing HF and non-HF patients) and risk stratification (distinguishing acute and chronic HF). The

potential correlation of salivary cortisol values with serum values was evaluated. Finally, the correlation of salivary cortisol values with the NT-proBNP (serum and/or salivary) values was also assessed.

2. METHODS

Patients with symptoms of dyspnoea and exercise intolerance were enrolled the clinical study, according to the protocol that wil be described in detail below:

2.1 Population

Three groups of patients were studied:

2.1.1 Acute HF (AHF)

Patients with acute heart failure (AHF), requiring hospital admission or outpatient intravenous (IV) diuretics, who were admitted in the 2nd Department of Cardiology of the University Hospital of Ioannina, consisted the **Group I** of the study. Those patients either had a first manefestation/ presentation of HF, i.e de novo HF, or they had already been diagnosed with HF and this presentation was an acute decompensation of a previously stable chronic heart failure (ADHF).

2.1.2 Chronic HF (CHF)

The second group of the study, **(Group II)** included patients with symptoms of HF and known HF, i.e. chronic stable HF (CHF) who were examined in the Heart Failure Outpatient Clinics of the 2nd Department of Cardiology of the University of Ioannina. Patients had a diagnosis of HF (with reduced, mildly reduced, or preserved EF) of any underlying aetiology, according to the latest European Society of Cardiology guidelines (2021).

2.1.3 Non-HF patients with dyspnea

This population served as the 'control population' and it consisted of patients with dyspnea that was not due to HF but other health problems that could explain the symptoms. It was sub-divided in two groups:

Group III: patients with dyspnea without HF, but with obesity, who were used as control subjects.

Group IV: patients with dyspnea without HF, but with hypertension, who were used as control subjects.

2.2 Eligibility Criteria

Inclusion criteria

Group I:

Patients with a diagnosis of AHF requiring hospital admission or outpatient intravenous (IV) diuretics.

Group II:

Patients with known stable HF. Patients who had a diagnosis of HF (with reduced, mildly reduced, or preserved EF) of any underlying aetiology, according to the latest European Society of Cardiology guidelines (2021).

Group III:

Patients with obesity with HF symptoms but no evidence of ventricular dysfunction on echocardiography or raised NPs. Obesity was defined as body mass index (BMI - calculated as weight in kilograms divided by height in meters squared) greater than 30 kg/m^2.

Group IV:

Patients with systemic arterial hypertension with HF symptoms but no evidence of ventricular dysfunction on echocardiography and normal or mildly raised NPs. Hypertension was defined as a consistent increase in systolic (SBP) or diastolic (DBP) blood pressure (SBP ≥140mmHg +/- DBP ≥90mmHg) or >1 month use of antihypertensive medications.

Exclusion criteria

- Patients positive to SARS-CoV-2 or recently recovered from COVID-19.
- Patients aged under 18 years of age
- Patients unable or unwilling to give informed consent
- Patients with cognitive, mental or psychiatric disorders
- Patients with alcohol or drug abuse

- Pregnant or breast-feeding female patients
- Patients on vegan and/or vegetarian diets
- Patients with a diagnosis (other than HF) compromising survival over the study period
- Severe co-morbidities such as:
  - Severe Chronic Obstructive Pulmonary Disease (e.g. patients on oxygen therapy or on nebulizers)
  - Uncontrolled dysthyroidism (Hyper/Hypo), i.e. TSH <0.03 or >7mIU/L
  - Decompensated Diabetes Melitus
  - Chronic Inflammatory Intestinal Diseases
  - Severe/Active Rheumatological Diseases
  - Active oncological disease on current or previous <1 year therapy of any kind
  - Liver failure
  - Severe kidney disease – eGFR<30 ml/min/1.73m^2

2.3 Data Collection, process, storage and management

Data collection activities span from November 2020 to March 2022.

All enrolled subjects underwent a thorough medical evaluation, the results of which were kept in a case report form **(Table 6 - Appendix)**. Apart from demographics, full medical history, family history, physical examination, electrocardiogram, echocardiogram, a questionnaire about their quality of life (EQ5D-5L – **Table 7**), blood and saliva samples were also collected from the patients, with procedures described in detail at the next sections.

Table 7. The EQ5D-5L QUESTIONNAIRE	
MOBILITY	
I have no problems in walking about	
I have slight problems in walking about	

I have moderate problems in walking about	
I have severe problems in walking about	
I am unable to walk about	
SELF-CARE	
I have no problems washing or dressing myself	
I have slight problems washing or dressing myself	
I have moderate problems washing or dressing myself	
I have severe problems washing or dressing myself	
I am unable to wash or dress myself	
USUAL ACTIVITIES (e.g. work, study, housework, family or leisure activities)	
I have no problems doing my usual activities	
I have slight problems doing my usual activities	
I have moderate problems doing my usual activities	
I have severe problems doing my usual activities	
I am unable to do my usual activities	
PAIN/DISCOMFORT	
I have no pain or discomfort	
I have slight pain or discomfort	
I have moderate pain or discomfort	
I have severe pain or discomfort	
I have extreme pain or discomfort	
ANXIETY/DEPRESSION	
I am not anxious or depressed	
I am slightly anxious or depressed	
I am moderately anxious or depressed	
I am severely anxious or depressed	

I am extremely anxious or depressed	

Study participants were assigned a study ID using a log. Data were entered initially into a paper case report form which recorded the study ID of the participant only, and was held in a secure location. The anonymised data from these forms were subsequently entered into a secure digital form for analysis.

For Group I patients, the investigators screened admissions each day for potentially eligible patients. If a potentially eligible patient was identified, as soon as their clinical condition was stabilized, the investigator approached that patient to inform them about the study and its main objectives, provided an information leaflet and invited them to participate. If a patient agreed to participate they were provided with a consent form, which was available in their local language, to consider and to sign if they wished to proceed. The consent form clearly stated that the participation was voluntary, that consent could be withdrawn and that all data acquired, would be anonymized.

Patients who agreed to participate underwent a standardized assessment that included the procedures described in **Table 8.**

Table 8: Overview of Study Evaluations in Group I

Activity	**Follow-up time point**		
	Day 0 (= T0)	Discharge (=T1)	Week 4 after discharge (=T4)
Log of suspected cases	X		
Eligibility assessment	X		
Selection (if necessary)	X		
Enrolment/Consenting	X		

Blood	X	X	X
Saliva	X	X	X
Demographic, clinical history, medication and risk factor assessment	X		X
MACE or hospitalisation since last visit			X
Physical examination	X	X	X
Vital signs at rest	X	X	X
ECG	X	X	X
Echocardiogram	X		X
6MWT	X if the patient could perform	X	X

For Group I Patients that were admitted to the hospital with acute dyspnea, a nasopharyngeal or pharyngeal test for SARS-CoV-2 would be obtained anyway, with all the appropriate precautions indicated by the Hospital. Blood and saliva samples were collected, provided that the patient had refrained from water and food consumption for the last two hours, preferably at the first morning of their hospitalization.

A 6MWT would be performed only if the patients was able to do it and a detailed echocardiogram would be performed as soon as the medical condition of the patient allowed it.

A second time-point for study collection was at the discharge of the patient and a third time-point (follow up) was scheduled about four weeks later. At the third time-point, the same procedures were followed, apart from the information sheet.

For Groups II, III and IV, all patients visiting the clinics of the participating institution were assessed for eligibility. If a potentially eligible patient was identified, the investigator approached that patient to inform them about the study, provide an information leaflet and invite them to participate. If a patient agreed to participate they were provided with a consent form, which was available in their local language, to consider and to sign if they wished to proceed. The consent form clearly stated that the participation was voluntary, that consent could be withdrawn and that all data acquired, would be anonymized.

Patients who agreed to participate underwent a standardized assessment that included the procedures described in **Table 9**. A nasopharyngeal or pharyngeal test for SARS-CoV-2 was acquired with all the appropriate precautions indicated by the Hospital. Blood and saliva samples were collected, provided that the patient had refrained from water and food consumption for the last two hours.

Table 9. Overview of Study Evaluations in Group II, III and IV

Activity	**Follow-up time point**	
	Day 0 (=T0)	Week 4 (=T4)
Log of invited cases	X	
Eligibility assessment	X	
Selection (if necessary)	X	
Enrolment/Consenting	X	
Specimen Collection		
Blood	X	X
Saliva	X	X
Clinical variables		
Demographic, clinical history, medication, and risk factor assessment	X	X
Major adverse cardiovascular		X

event or hospitalisation since last visit		
Physical examination	X	X
Vital signs at rest	X	X
ECG	X	X
Echocardiogram	X	Unless clinical situation changed within previous 1 month.
6MWT	X	X

After the samples were acquired, a detailed physical examination would follow. The medical history with all the medications was taken, the family history, the patients' habits and lifestyle were recorded, an electrocardiogram was recorded, a 6MWT and an echocardiogram were performed.

All these data were acquired in a quiet environment, friendly to the patients, in order to make them feel comfortable. All these tests were performed in a single visit (approximately 2 hours per patient).

A telephone visit was scheduled in order to inform the patient about the results of the examination (blood etc) and possible treatment modifications; a second visit (follow up) was scheduled about four weeks later. At the second visit, the same procedures were followed, apart from the information sheet. Moreover, a second echocardiogram was not performed, unless there was a medical indication for that, i.e. a change in clinical status (mainly for the Group II patients).

2.4 Saliva collection. Saliva biomarkers.

Saliva samples were collected with a commercially available kit. Briefly, a SalivaBio Oral Swab (SOS) was used to collect stimulated saliva from HF patients **(Figure 3)**.

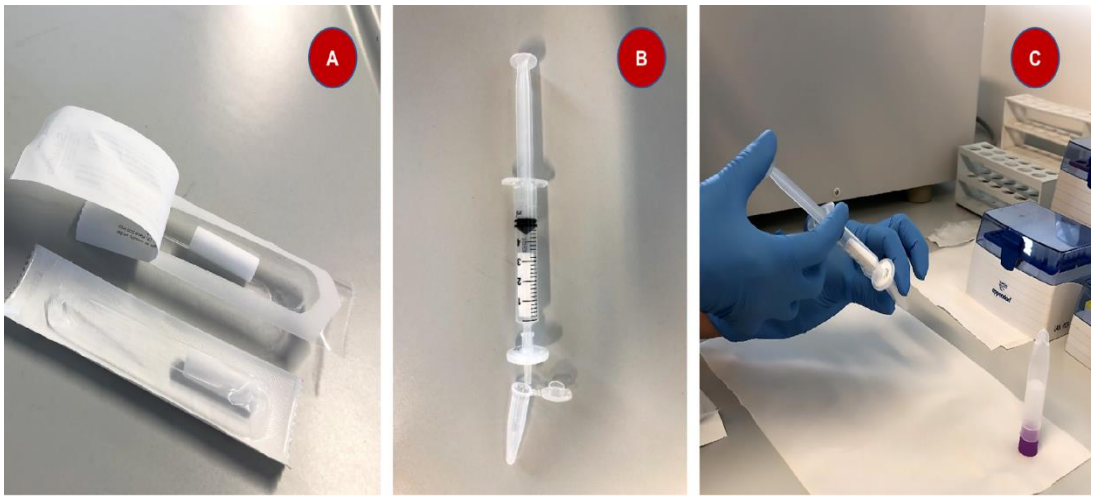


Figure 3. *An oral swab (a) was placed in the space between the buccal wall and the oral gingival or beneath the tongue, for two minutes. The swab was then collected with sterile gloves and placed in a syringe, at the tip of which a filter was placed to remove any content (b). The saliva sample was infused in a plastic tube (c) to be centrifuged at 4000rpm for two minutes, before storage at -20^{o}C.*

Stimulated saliva was produced as the salivary glands were triggered by physical stimulation like chewing. Samples were collected between 8 and 10am in order to minimize variations of sample composition related to the circadian rhythms. Each patient was asked to refrain from eating, drinking, smoking, chewing gum and oral hygiene practices for at least 1 hour prior to saliva collection. Saliva speciments were maintained at -20^{o}C and then measured by ELISA kits for determining salivary NT-proBNP and Cortisol.

It has to be noted that, originally the study protocol was drafted before the SARS-CoV-2 pandemic occurred, and therefore it had to be modified due to the potential transmission of an upper respiratory tract infection of the virus with the saliva collection. This section provides the procedures that were followed for the saliva samples during the pandemic for each study group.

For patients with AHF (Group I):

Patients admitted at the hospital with acute dyspnoea were treated as potential COVID-19 patients, and a nasopharyngeal or pharyngeal swab test was performed to rule out SARS-CoV-2 infection. At the time of admission, participation in the project

was proposed to the patient who was requested to sign an informed consent form. After consent was given and as soon as possible after the admission,, the saliva sample was collected with the SOS device (with all necessary precautions in place). While waiting for the result of the (naso)pharyngeal test, the sample was considered infected and was packaged according to the hospital safety procedures and stored at -20^{0}C. In case of a negative SARS-CoV-2 test, the saliva sample was considered "COVID free" and it was stored at -20^{0}C to avoid any temperature variations during the storage. During hospitalization, the patient was isolated and could not have any visitors; therefore all samples taken during this period were considered COVID free. An additional saliva sample was collected at discharge. Immediately after collection, this sample was frozen as described above.

A follow-up visit was set about four weeks after discharge. An additional saliva sample was taken, and a test was performed again for COVID-19. If the test was negative, the last sample taken was considered "COVID free" and then was transferred for storage.

In case of a positive test for COVID-19 in any of the phases described above, the patient would be excluded from the study and all the samples taken up to that moment would be destroyed according to the predefined hospital procedures.

For patients with CHF (Group II), obesity or hypertension (Groups III, IV):

At the time of the visit to the hospital outpatient clinic, participation in the project was proposed to the patient who was asked to sign an informed consent form; a saliva sample was taken with the SOS device, and a test was performed to rule out SARS-CoV-2 infection. While waiting for the result of the COVID test, the sample was considered infected and was packaged according to the hospital safety procedures, and stored at -20°C.

In case of negative SARS-CoV-2 test, the saliva sample was considered "COVID free", and it was transferred and stored at -20^{0}C to avoid any temperature variations during the storage.

A follow-up visit was set about four weeks later. An additional saliva sample was taken and another test was performed for SARS-CoV-2, following the same

procedure described for the previous sample. If the test was negative, the sample was considered “COVID free”, and it was transferred and stored as described above.
In case of a positive test for COVID-19 in any of the phases described above, the patient would be excluded from the study and all the samples taken up to that moment would be destroyed according to the predefined hospital procedures.

2.5 Blood collection. Blood biomarkers

Blood was drawn from venepuncture with standard procedures, with particular attention to proper technique. Approximately 25ml of blood was used in every sample collection at every time-point of the study. Plasma NT-proBNP was measured using standard POC devices or biochemistry laboratory and plasma cortisol was measured using biochemistry laboratory. After the procedure the patients were monitored for 15 minutes and if stable and asymptomatic they continued with the rest of the procedures.

2.6 Six minute walking test

The six minute walking test (6MWT) is a sub-maximal exercise test used to assess aerobic capacity and endurance (493). The examination was performed in an even straight 30-meter long corridor, with adequate lighting, which is isolated from other clinics so that there would be no disruptions for the patient. Vital signs (arterial blood pressure, heart rate and oxygen saturation) were recorded at the beginning of the test. Then the patient would walk as quickly as possible, one or two steps ahead of the investigator. The investigator would encourage the patient to continue and assess for symptoms. A chair was available at the corridor in case the patient would like to rest (with the time running). When the 6 minutes passed, the vital signs would be monitored again and the patient would be asked for symptoms.

We evaluated the distance covered over a time of 6 minutes (which is indicative of the performance capacity) and compared any changes in the vital signs prior to and after the test.

2.7 Echocardiogram

The echocardiogram was a fundamental examination for the study both for the diagnosis or exclusion of HF, but also for the clinical outcome and improvement of AHF patients prior to discharge. Group I patients had 2 echocardiograms (timepoint 0 – admission, and one-month follow-up), while patients from Group II,III, and IV had one echocardiogram. Philips Affiniti 50G, S4-2 and Philips Sparq ultrasound systems were used.

For Group I patients, a thorough bedside evaluation took place as soon as they were admitted, or after stabilization in case of medical emergency. If the patient was admitted at the cardiac intensive care unit the Philips Sparq would be used, and if admitted at the 2nd Cardiology Department, the Philips Affiniti 50G, S4-2 system would be used.

For Group II,III, and IV the Affiniti 50G, S4-2 would be used, at the Outpatients Clinic. The echocardiography device was located in a semidark room, with quiet environment, while a dedicated nurse would prepare the patient for the exam.

In all cases, the exam initially contained personalized data for clinical evaluation and reproducibility reasons, but all images were deanonymized before extracted and stored for research purposes

2.8 Quality of life questionnaire (EQ-5D-5L)

The EQ-5D-5L questionnaire essentially consists of 2 sections (one per page): the EQ-5D descriptive system and the EQ visual analogue scale (EQ VAS) (**Table 7**). The descriptive system comprises five dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression), each one having 5 levels (no problems, slight problems, moderate problems, severe problems and extreme problems). The patient subjectively rates his/her status in each dimension by choosing the most appropriate statement. The EQ VAS records the patient's self-rated olistic health status, on a vertical visual analogue scale (from the worst to the best health they can image for themselves). It is important to note that the questionnaire we used has been previously validated in order to be used in the Greek population (494).

All patients participating in the study completed the questionnaire at each timepoint, usually after the completion of all other procedures, so that they would be more familiar and feel more comfortable with the investigator. The completion took place as a face-to-face interview in their native language, in a quiet office.

2.9 Timepoints of the study

There are three timepoints that the patients where studied and data were collected. The first is Timepoint 0 (T0), i.e. the first day that each patient was included in the study. For Group I, T0 is the day that the patient was admitted in the hospital. Timepoint 1 (T1) applies only in Group 1 patients and is the day of their discharge from hospital. Timepoint 4 (T4) applies for all study units and is the follow-up examination, 4 weeks after T1 for Group 1 patients and 4 weeks after T0 for Group II, III, IV patients. The above nomnclature will be followed in all tables and in the Results' section.

2.10 Statistical Analysis

For the comparison of two independent samples, the Independent-Samples T-Test was used. The normality was assessed using the Shapiro-Wilk criteria and the extreme values (outliers) should be less than 10%. In cases where one of the two requirements was not met, or when asymptotic normality could not be assumed, non parametric tests like the Mann-Whitney, wereused.

The Paired-Samples T-Test was used for the comparison of two dependent samples. The normality was assessed using the Shapiro-Wilk criteria and the extreme values should be less than 10%. In cases where one of the two requirements was not met, or when asymptotic normality could not be assumed, non parametric tests like the Wilcoxon, were used.

Especially for the categorical variable NYHA, that has more than two options, the analysis of variance (ANOVA) was used. The normality in each sub group was

assessed using the Shapiro-Wilk criteria and the extreme values should be less than 10%. In cases where one of the two requirements was not met, or when asymptotic normality could not be assumed, non parametric tests like the Kruskal-Wallis, were used.

The Pearson's chi-squared test was applied to sets of categorical data, provided that the number of observations was four times larger than the product of the number of sub-groups of the two variables and when the percentage of expected frequencies were less than five; these should not exceed 25%.

Finally for the linear correlation between two quantitative variuables, the Pearson correlation coefficient was used. The normality was assessed using the Shapiro-Wilk criteria and the extreme values should be less than 10%. In cases where one of the two requirements was not met, or when asymptotic normality could not be assumed, the non parametric Spearman's correlation coefficient was used.

The analysis was performed with the SPSS v21.0 Software, and the level of statistical significance was determined equal to 0.05 in all cases.

3 RESULTS

3.1 Descriptive characteristics of the study population.

The descriptive characteristics of the four groups that consist the study population are shown in **Table 10**. All comparisons were made at baseline (time point 0 - T0). The HF population (i.e. Groups I and II) were older compared to non HF-patients (Groups III and IV) and mostly males. Other differences between groups will be presented further below.

Table 10. Descriptive statistics at baseline (time point 0)

	Acute HF, n=49 Group I	**Chronic HF, n=12 Group II**	**Obese, n=18 Group III**	**Hypertensive, n=18 Group IV**
Age, years*	72±12	72±9	50±11	65±10
Male gender, n (%)	35 (70)	7 (54)	8 (44)	9 (50)
CV risk factors, n (%)				
Hypertension	43 (86)	10 (77)	5 (28)	18 (100)
Dyslipidemia	38 (76)	11 (85)	7 (39)	15 (83)
Diabetes	25 (50)	4 (31)	0 (0)	3 (17)
Obesity	11 (22)	4 (31)	18 (100)	6 (33)
Smoking	6 (12)	1 (8)	6 (33)	3 (17)
History of, n (%)				
CAD	22 (44)	5 (39)	0 (0)	3 (17)
Stroke/TIA	6 (12)	1 (8)	0 (0)	1 (6)
PAD	4 (8)	1 (8)	0 (0)	0 (0)
CKD	23 (46)	3 (25)	1 (6)	1 (6)
AF	29 (58)	6 (50)	1 (6)	1 (6)
Medications, n (%)				
ACEi	8 (16)	3 (25)	0 (0)	1 (6)
ARB	11 (22)	4 (33)	1 (6)	12 (67)
ARNI	5 (10)	1 (8)	0 (0)	0 (0)
CCB	8 (16)	6 (50)	2 (11)	10 (56)
Statin	29 (58)	9 (69)	3 (17)	15 (83)
B-blocker	34 (68)	8 (67)	4 (22)	7 (39)

MRA	26 (52)	5 (39)	1 (6)	1 (6)
Loop diuretic	37 (74)	8 (67)	1 (6)	0 (0)
Thiazide diuretic	0 (0)	2 (16)	0 (0)	6 (33)
Anticoagulant	31 (62)	5 (39)	0 (0)	0 (0)
Antiplatelet	15 (30)	5 (39)	0 (0)	1 (6)
SBP, mmHg*	130±25	133±16	139±14	149±22
DBP, mmHg*	70±14	67±11	84±11	87±13
BMI, kg/m^2*	27.2±4.8	28.6±4.0	35.1±3.5	29.9±4.4
6MWD, m*	263±151	362±165	531±121	492±103
Hb, g/dl*	12.5±2.2	13.3±2.3	14.3±1.9	14.3±1.2
Glucose, mg/dl**	138 (106, 168)	98 (90, 157)	94 (90, 106)	101 (94, 107)
eGFR, ml/min/1.73m^2*	55.1±18.5	64.5 ±17.4	82.2±22.9	80.0±13.5
TCHOL, mg/dl*	154±36	184 ± 52	219±31	180±53
HDL, mg/dl*	43±13	48±13	55±10	55±13
TRG, mg/dl*	125±65	129±63	113±44	109±42
LDL, mg/dl*	85±29	111±43	142±33	104±40^{+}
Echo parameters*				
LVEF, %	38±19	45±15	60±2	60±3
LVIDD, mm	57±12	52±10	48±5	43±6
RWT	0.42±0.16	0.47±0.13	0.42±0.09	0.54±0.12
LVH, n (%)	31 (62)	6 (46)	4 (22)	7 (39)
E, cm/s	102±42	99±27	76±19	69±14
E/E'**	15.0 (12.8, 16.0)	12.0 (8.8, 13.5)	7.5 (6.8, 9.0)	7.0 (6.9, 9.0)
TRVmax, m/s	3.0±0.8	2.6±0.7	2.1±0.6	1.9±0.5
SerumNT-proBNP**	4706 (1237, 8438)	1232 (566, 3509)	64 (60, 88)	98 (63, 122)
Saliva NT-proBNP**	15.1 (10.4, 30.6)	8.6 (7.1, 35.2)	8.8 (6.3, 22.7)	11.1 (6.0, 17.6)
Serum Cortisol**	21.26 (2.2, 61.4)	10.14 (1.6, 23.3)	9.82 (5.7, 17.2)	10.89 (5.7, 22.3)
Saliva Cortisol**	5.62 (0.05, 15.63)	6 (0.37, 14.82)	4.07 (0.41, 11.08)	3.2 (0.98, 12.94)

*mean ± standard deviation **median (IQR)

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin

receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

At first, we compared the serum NT-proBNP values (gold standard biomarker) at time point 0, for Groups III and IV. The reason for this was to assess whether we could combine those two groups of patients and create a single control group (defined as the non-HF population), or these should be considered as two separate groups that should be compared with the HF population (Groups I and II). The size of the two samples was small (18 each) and normality test conducted in both groups showed deviation from normality. The Mann Whitney (non parametric) test revealed a statistically significant difference in the median values of the two groups (p=0.025) with lower values found in obese compared to hypertensive patients. As as result, we continued the analysis, considering the two groups separately. However, it should be noted that obesity is known to be associated with lower than expected NT-proBNP values [65] and thus this difference between the 2 groups could be attributed to obesity.

	Obese n=18 Group III	Hypertensive n=18 Group IV	P value
Serum NT-proBNP, pg/mL median(IQR)	64 (60, 88)	98 (63, 122)	0.025

Additionally, statistically significant differences were found in the median values of these two groups in the following variables: age, weight, BMI, serum urea, total cholesterol, LDL cholesterol, left ventricle internal diastolic diameter, left ventricle regional wall thickness, and diastolic mitral flow pattern (E/A ratio) (**Table 11)**. Mann

Whitney U test was used due to deviation from normality. A worse lipid profile, greater left ventricular dimensions, a lower regional wall thickness and higher E/A ratio was found in obese compared to hypertensive individuals.

Table 11. Variables for which statistically significant differences were found between Group III and Group IV

	Obese n=18 Group III	Hypertensive n=18 Group IV	**P value**
Age, years*	50±11	65±10	**0.001**
Weight, kg*	102±17	88±18	**0.012**
BMI,*	35.1±3.5	29.9±4.4	**0.0001**
Urea, mg/dL*	27.5±7	36.5±10	**0.002**
Cholesterol, mg/dL*	219±31	180±53	**0.027**
LDL, mg/dL*	142±33	104±40	**0.008**
LVIDD, mm*	48±5	43±6	**0.025**
RWT*	0.42±0.09	0.54±0.12	**0.0001**
E/A ratio*	1.05±0.32	0.79±0.2	**0.006**
Serum NT-proBNP, pg/mL**	64 (60, 114)	96 (60,186)	**0.025**

BMI= body mass index, LDL= low-density lipoprotein cholesterol, LVIDD= left ventricle internal diastolic diameter, RWT= regional wall thickness. *mean±SD, **median (IQR)

We then compared the Obese and Hypertensive patients (Group III and IV) with the CHF patients (Group II) (see **Table 12)**. Hypertensive and CHF patients were older compared to obese patients ($p<0.05$ for both). There were various differences in the prevalence of CVD and the use of medications between the 3 groups; as expected, CHF patients had a higher prevalence of CAD, AF and impaired renal function and received more anti-HF medications, while hypertensive patients received more anti-hypertensive medications. The lowest utilization rate of medications was found in the obese group. Hypertensive and obese patients walked a greater distance in

6MWT compared to CHF patients (p<0.05 for both), had a higher value of LVEF, a larger left ventricle, higher left ventricular filling pressures (as indicated by the E/E' ratio) and estimated systolic pulmonary artery pressures (as indicated by the TR maximal velocity) (p<0.05 for all) at T0. Finally, serum NT-proBNP levels were statistically different among the groups, with CHF patients having higher values [1232pg/mL (566, 3509) vs 64pg/mL (60, 88) vs 98pg/mL (63, 122)]. However, this difference was not found in the saliva results. **(Table 12)**.

Table 12. Comparison among Obese, Hypertensive and CHF patients (Groups II, III, IV) at timepoint 0

	Chronic HF, n=12 Group II	**Obese, n=18 Group III**	**Hypertensive, n=18 Group IV**	**P value, Kruskal Wallis test**
Age, years*	72±9	50±11	65±10	**<0.001**
Male gender, n (%)	7 (54)	8 (44)	9 (50)	0.870
CV risk factors, n (%)				
Hypertension	10 (77)	5 (28)	18 (100)	**<0.001**
Dyslipidemia	11 (85)	7 (3)	15 (83)	**0.005**
Diabetes	4 (31)	0 (0)	3 (17)	0.051
Obesity	4 (31)	18 (100)	6 (33)	**<0.001**
Smoking	1 (8)	6 (33)	3 (17)	0.265
History, n (%)				
CAD	5 (39)	0 (0)	3 (17)	**0.017**
Stroke/TIA	1 (8)	0 (0)	1 (6)	0.522
PAD	1 (8)	0 (0)	0 (0)	0.243
CKD	3 (23)	1 (6)	1 (6)	**0.012**
AF	6 (46)	1 (6)	1 (6)	**0.003**
Medications, n (%)				
ACEi	3 (23)	0 (0)	1 (6)	0.06

ARBs	4 (31)	1 (6)	12 (67)	**0.001**
CCB	6 (46)	2 (11)	10 (56)	**0.016**
Statins	9 (69)	3 (17)	15 (83)	**<0.001**
B-blockers	8 (62)	4 (22)	7 (39)	0.086
MRA	5 (39)	1 (6)	1 (6)	**0.015**
Loop diuretics	8 (62)	1 (6)	0 (0)	**<0.001**
Thiazide diuretics	2 (15)	0 (0)	6 (33)	**0.026**
SBP, mmHg*	133±16	139±14	149±22	**0.045**
DBP, mmHg*	67±11	84±11	87±13	**<0.001**
BMI, kg/m^2*	28.6±4.0	35.1±3.5	29.9±4.4	**<0.001**
6MWD, m*	362±165	531±121	492±103	**0.002**
Hb, g/dl*	13.3±2.3	14.3±1.9	14.3±1.2	0.220
Glucose, mg/dl**	98 (90, 157)	94 (90, 106)	101 (94, 107)	0.421
eGFR, ml/min/1.73m^2*	64.5±17.4	82.2±22.9	80.0±13.5	**0.026**
TCHOL, mg/dl*	184±52	219±31	180±53	**0.032**
HDL, mg/dl*	48±13	55±10	55±13	0.226
TRG, mg/dl*	129±63	113±44	109±42	0.505
LDL, mg/dl*	111±43	142±33	104±40	**0.032**
Echo parameters*				
LVEF, %	45±15	60±2	60±3	**<0.001**
LVIDD, mm	52±10	48±5	43±6	**0.004**
RWT	0.47±0.13	0.42±0.09	0.54±0.12	**0.011**
LVH, n (%)	6 (46)	4 (22)	7 (39)	0.345
E, cm/s	99±27	76±19	69±14	**0.001**
E/E'**	12.0 (8.8, 13.5)	7.5 (6.8, 9.0)	7.0 (6.9, 9.0)	**<0.001**
TRVmax, m/s	2.6±0.7	2.1±0.6	1.9±0.5	**0.009**
Serum NT-	1232 (566,	64 (31, 93)	106 (63, 120)	**<0.001**

proBNP**	3509)			
Saliva NT-proBNP**	8.6 (7.1, 35.2)	8.8 (6.3, 22.7)	11.1 (6.0, 17.6)	0.969
*mean±Standard Deviation, **median (IQR)				

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

Comparing AHF patients (Group I) and CHF (Group II), no significant differences were found in age, gender, CV risk factors, cardiovascular disease history, or medications between the two groups (p=NS for all, with the exception of calcium channel blockers' use) **(Table 13).** Higher serum NT-proBNP values [4706pg/mL (1237, 8438) vs 1232pg/mL (566, 3509)], NYHA class and lower 6MWD values were observed in AHF compared to CHF patients (p<0.05 for both). Increased E/E' and inferior vena cavae diameter in expiration (IVC exp) were found in ADHF vs CHF patients (p<0.05 for both, **Table 13**), indicating increased congestion in ADHF patients. ADHF patients had higher glucose, urea, total and LDL cholesterol compared to CHF patients (p<0.05 for all, **Table 13**).

Table 13. Comparison among AHF and CHF patients (Groups I, II)

	Chronic HF, n=12 Group II	**Acute HF, n=49 Group I**	**P value, Mann Whitney U test**
Age, years*	72±9	72±12	0.981
Male gender, n (%)	7 (54)	35 (70)	0.329
CV risk factors, n (%) Hypertension	10 (77)	43 (86)	0.417

Dyslipidemia	11 (85)	38 (76)	0.714
Diabetes	4 (31)	25 (50)	0.349
Obesity	4 (31)	11 (22)	0.489
Smoking	1 (8)	6 (12)	0.882
CVD History, n (%)			
CAD	5 (39)	22 (44)	0.764
Stroke/TIA	1 (8)	6 (12)	0.660
PAD	1 (8)	4 (8)	0.971
CKD	3 (23)	23 (46)	0.207
AF	6 (46)	29 (58)	0.537
Medications, n (%)			
ACEi	3 (23)	8 (16)	0.549
ARBs	4 (31)	11 (22)	0.508
ARNI	1 (8)	5 (10)	0.801
CCB	6 (46)	8 (16)	**0.020**
Statins	9 (69)	29 (58)	0.538
B-blockers	8 (62)	34 (68)	0.660
MRA	5 (39)	26 (52)	0.536
Loop diuretics	8 (62)	37 (74)	0.376
Anticoagulation	5 (39)	31 (62)	0.208
Antiplatelets	5 (39)	15 (30)	0.559
SBP, mmHg*	133±16	130±25	0.628
DBP, mmHg*	67±11	70±14	0.446
HR, bpm*	73±20	79±16	0.265
BMI, kg/m^2*	28.6±4.0	27.2±4.8	0.331
6MWD, m*	362±165	263±151	**0.045**
NYHA class (n%)			
I	1 (8)	0 (0)	

II III IV	3 (23) 8 (61) 1 (8)	1 (2) 30 (60) 19 (38)	**0.003**
QRS, msec**	118 (90, 159)	140 (99, 158)	0.535
WBC*	7238±2059	8499±2832	0.139
Hb, g/dl*	13.3±2.3	12.5±2.2	0.261
Glucose, mg/dl*	98 (90, 157)	138 (106, 168)	**0.036**
Urea, mg/dl*	51 (39, 80)	73 (54, 101)	**0.045**
eGFR, ml/min/1.73m^2*	64.5±17.4	55.1±18.5	0.107
K+*	4.05±0.41	4.24±0.52	0.216
Na+**	139 (138, 141)	138 (135, 140)	0.189
TCHOL, mg/dl*	184±52	154±36	**0.016**
HDL, mg/dl*	48±13	43±13	0.281
TRG, mg/dl*	129±63	125±65	0.844
LDL, mg/dl*	111±43	85±29	**0.013**
Echo parameters* LVEF, % LVIDD, mm RWT LVH, n (%) E, cm/s E/E'** TRVmax, m/s IVCexp, mm	 45±15 52±10 0.47±0.13 6 (46) 99±27 12.0 (8.8, 13.5) 2.6±0.7 13 (10, 19)	 38±19 57±12 0.42±0.16 31 (62) 102±42 15.0 (12.8, 16.0) 3.0±0.8 23 (20, 25)	 0.206 0.176 0.366 0.353 0.775 **0.018** 0.131 **0.001**
Serum NT-proBNP**	1232 (566, 3509)	4706 (1237, 8438)	**0.007**
Saliva NT-proBNP**	8.6 (7.1, 35.2)	15.1 (10.4, 30.6)	0.122
*mean ± standard deviation**median (IQR)			

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

3.2 Serum and salivary NT-proBNP comparisons among study groups at various timepoints.

Regarding the NT-proBNP values in both serum and saliva samples, we compared their values at different timepoints (day of admission or day of first exam = T0, day of discharge = T1 only for AHF patients, 4 week follow-up = T4, for all) within each group for all groups (acute - AHF, chronic - CHF, obese - OB, hypertensive - HT). We also compared the values between different groups for the same timepoints. As tests of normality failed (i.e. when the percentage of extreme values in one or both samples is more than 10% or when populations do not have normal distributions), non-parametric tests (Mann Whitney or Wilcoxon for comparison of independent and paired samples respectively) were used. All comparisons are shown in **Table 14**.

The serum values of NT-proBNP in AHF patients were statistically significantly higher when compared to all other study groups (Mann Whitney U test) both at T0 and at T4. A statistically significant difference was also found between T0 and T1 serum NT-proBNP for AHF patients (i.e.higher at baseline compared to discharge), but not between T1 and T4 (i.e. between discharge and 4-week follow-up).

The serum values of NT-proBNP did not differ between timepoints 0 and 4 in any of the stable groups patients with either CHF, obesity or hypertension, indicating a satisfactory repeatability of the measurement, although this was not formally evaluated in our study.

The serum values of NT-proBNP in CHF patients were statistically significant higher when compared to Groups III and IV (obese and hypertensive) both at T0 and T4, as

expected. Similarly, the serum values of NT-proBNP in thw combined Group I and II (all HF patients) were statistically significantly lower when compared to Groups III and IV, both at timepoints 0 and 4.

Table 14. Comparison between serum NT-proBNP median (IQR) values among study groups and at all timepoints					
Variable 1	**Median (IQR)**	**Variable 2**	**Median (IQR)**	**Test**	**P value**
ALL Serum NT-proBNP at T0	844 (4810)	ALL Serum NT-proBNP at T4	527 (2401)	Wilcoxon, Signed Rank Test	0.158
AHF Serum NT-proBNP at T0	4493 (7136)	AHF Serum NT-proBNP at T1	2681.5 (5671)	Wilcoxon, Signed Rank Test	**0.002**
AHF Serum NT-proBNP at T0	4493 (7136)	AHF Serum NT-proBNP at T4	2873.5 (5442)	Wilcoxon, Signed Rank Test	0.061
AHF Serum NT-proBNP at T1	2681.5 (5671)	AHF Serum NT-proBNP at T4	2873.5 (5442)	Wilcoxon, Signed Rank Test	0.994
AHF Serum NT-proBNP at T0	4493 (7136)	CHF Serum NT-proBNP at T0	1221.5 (1818)	Mann Whitney U Test	**0.007**
AHF Serum NT-proBNP at T0	4493 (7136)	OB Serum NT-proBNP at T0	64 (33)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T0	4493 (7136)	HT Serum NT-proBNP at T0	110 (118)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T0	4493 (7136)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
AHF+CHF Serum NT-proBNP at T0	3826 (6213)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T0	1221.5 (1818)	CHF Serum NT-proBNP at T4	1115(1394)	Wilcoxon, Signed Rank Test	1.000
CHF Serum NT-proBNP at T0	1221.5 (1818)	OB Serum NT-proBNP at T0	64 (33)	Mann Whitney U Test	**0.001**

CHF Serum NT-proBNP at T0	1221.5 (1818)	HT Serum NT-proBNP at T0	110 (118)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T0	1221.5 (1818)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	CHF Serum NT-proBNP at T4	1115(1394)	Mann Whitney U Test	**0.047**
AHF Serum NT-proBNP at T4	2873.5 (5442)	OB Serum NT-proBNP at T4	60.5 (63)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	HT Serum NT-proBNP at T4	112.5 (125)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
AHF+CHF Serum NT-proBNP at T4	2142.5 (4994)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	OB Serum NT-proBNP at T4	60.5 (63)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	HT Serum NT-proBNP at T4	112.5 (125)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
OB Serum NT-proBNP at T0	64 (33)	OB Serum NT-proBNP at T4	60.5 (63)	Wilcoxon, Signed Rank Test	0.625
HT Serum NT-proBNP at T0	110 (118)	HT Serum NT-proBNP at T4	112.5 (125)	Wilcoxon, Signed Rank Test	0.691
OB+HT Serum NT-proBNP at T0	75.5 (64)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Wilcoxon, Signed Rank Test	0.919

AHF+CHF Serum NT-proBNP at T0	3826 (6213)	AHF+CHF Serum NT-proBNP at T4	2142.5 (4994)	Wilcoxon, Signed Rank Test	0.086

AHF = acute HF; ALL = Total study population; CHF= chronic HF; HT=hypertensive; OB=obese; T0=day of admission or day of first exam; T1=day of discharge for AHF patients; T4 = four week follow-up.

Concerning the saliva biomarker, all comparisons are shown in **Table 15.** The saliva values of NT-proBNP did not differ between timepoints 0 and 4 in any of the groups. In this case, whether this indicates repeatability or lack of statistical power cannot be resolved here. Of note, the saliva values of the biomarkers are significantly lower compared to the serum values; this, taken into account their variability (as reflected in the IQR of the median), indicates that larger samples would be needed to show any significant difference. Furthermore, no statistically significant difference was found either between T0 and T1 saliva NT-proBNP for AHF patients (i.e.baseline compared to discharge), or between T1 and T4 (i.e. between discharge and 4-week follow-up), in contrast to was found in the serum levels of the biomarker. Similarly, the salivary values of NT-proBNP in CHF patients showed no statistically significant difference to any other group, in contrast to the serum biomarker values.

However, the salivary values of NT-proBNP in AHF patients were statistically significantly higher when compared to Group IV patients (hypertensive) as well as the combined Group III and IV (obese and hypertensive) at T0 and T4, probably as expected, but not when compared to obese alone or CHF patients, at any time point. Moreover, the combined Group I and II (all HF patients) salivary values of NT-proBNP were statistically significantly higher when compared to Groups III and IV, both at timepoints 0 and 4, also as expected.

Table 15. Comparison between salivary NT-proBNP median (IQR) values among study groups and at all timepoints.					
Variable 1	**Median (IQR)**	**Variable 2**	**Median (IQR)**	**Test**	**P value**
ALL Saliva NT-proBNP at T0	12.7 (16.3)	ALL Saliva NT-proBNP at T4	10.6 (0)	Wilcoxon, Signed Rank Test	0.354
AHF Saliva NT-proBNP at T0	15.1 (18.3)	AHF Saliva NT-proBNP at T1	10.7 (0)	Wilcoxon, Signed Rank Test	0.13
AHF Saliva NT-proBNP at T0	15.1 (18.3)	AHF Saliva NT-proBNP at T4	14.3 (24)	Wilcoxon, Signed Rank Test	0.457
AHF Saliva NT-proBNP at T1	10.7 (0)	AHF Saliva NT-proBNP at T4	14.3 (24)	Wilcoxon, Signed Rank Test	0.727
AHF Saliva NT-proBNP at T0	15.1 (18.3)	HT Saliva NT-proBNP at T0	11.1 (11.6)	Mann Whitney U Test	**0.032**
AHF Saliva NT-proBNP at T0	15.1 (18.3)	CHF Saliva NT-proBNP at T0	8.2 (0)	Mann Whitney U Test	0.162
AHF Saliva NT-proBNP at T0	15.1 (18.3)	OB Saliva NT-proBNP at T0	8.8 (0)	Mann Whitney U Test	0.062
AHF Saliva NT-proBNP at T0	15.1 (18.3)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	**0.012**
AHF+CHF Saliva NT-proBNP at T0	14.3 (22.3)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	**0.028**
AHF Saliva NT-proBNP at T4	14.3 (24)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Mann Whitney U Test	**0.037**
AHF Saliva NT-proBNP at T4	14.3 (24)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	**0.031**
AHF+CHF Saliva NT-proBNP at T4	13.8 (24.6)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	**0.033**

CHF Saliva NT-proBNP at T0	8.2 (0)	CHF Saliva NT-proBNP at T4	8.2 (0)	Wilcoxon, Signed Rank Test	0.674
OB Saliva NT-proBNP at T0	8.8 (0)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Wilcoxon, Signed Rank Test	0.446
HT Saliva NT-proBNP at T0	11.1 (11.6)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Wilcoxon, Signed Rank Test	0.619
OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Wilcoxon, Signed Rank Test	0.451
AHF+CHF Saliva NT-proBNP at T0	14.3 (22.3)	AHF+CHF Saliva NT-proBNP at T4	13.8 (24.6)	Wilcoxon, Signed Rank Test	0.527
CHF Saliva NT-proBNP at T0	8.2 (0)	OB Saliva NT-proBNP at T0	8.8 (0)	Mann Whitney U Test	0.851
CHF Saliva NT-proBNP at T0	8.2 (0)	HT Saliva NT-proBNP at T0	11.1 (11.6)	Mann Whitney U Test	0.819
CHF Saliva NT-proBNP at T0	8.2 (0)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	0.812
AHF Saliva NT-proBNP at T4	14.3 (24)	CHF Saliva NT-proBNP at T4	8.2 (0)	Mann Whitney U Test	0.928
AHF Saliva NT-proBNP at T4	14.3 (24)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Mann Whitney U Test	0.168
CHF Saliva NT-proBNP at T4	8.2 (0)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Mann Whitney U Test	0.495
CHF Saliva NT-proBNP at T4	8.2 (0)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Mann Whitney U Test	0.397

CHF Saliva NT-proBNP at T4	8.2 (0)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	0.393

AHF = acute HF; ALL = Total study population; CHF= chronic HF; HT=hypertensive; OB=obese; T0=day of admission or day of first exam; T1=day of discharge for AHF patients; T4 = four week follow-up.

Concerning the linear correlations between serum and salivary NT-proBNP, we ran tests for every meaningful combination for all study groups and at all timepoints **(see Table 16).** When the percentage of extreme values in one or both samples was more than 10% or when populations did not have normal distributions, non parametric tests (Mann Whitney, Wilcoxon, Kruskal and Wallis) were used. A statistically significant linear correlation ($p<0.05$) between serum and salivary NT-proBNP was found only in 4 cases: for the whole study population at T0, for Group I (AHF) at T0 and T1 and for the combined Group I and II (all HF population) at T0. In these 4 cases, it may be observed that either the samples were large or the biomarker values were high. However, it should be noted that the R value was moderate to weak in all significant corelations.

Table 16. Linear correlation between serum and salivary NT-proBNP mean values

Variable 1	Variable 2	Correlation Coefficient	R value	P value
ALL Serum NT-proBNP at T0	ALL Saliva NT-proBNP at T0	Spearman	**0.336**	**0.001**
ALL Serum NT-proBNP at T4	ALL Saliva NT-proBNP at T4	Spearman	0.170	0.118
AHF Serum NT-proBNP at T0	AHF Saliva NT-proBNP at T0	Spearman	**0.341**	**0.016**
AHF Serum NT-proBNP at T1	AHF Saliva NT-proBNP at T1	Pearson	**0.399**	**0.005**
AHF Serum NT-proBNP at T4	AHF Saliva NT-proBNP at T4	Pearson	-.093	0.557
AHF+CHF Serum NT-proBNP at T0	AHF+CHF Saliva NT-proBNP at T0	Pearson	**0.306**	**0.016**
AHF+CHF Serum NT-proBNP at T4	AHF+CHF Saliva NT-proBNP at T4	Pearson	-.052	0.721
CHF Serum NT-proBNP at T0	CHF Saliva NT-proBNP at T0	Spearman	0.294	0.354
CHF Serum NT-proBNP at T4	CHF Saliva NT-proBNP at T4	Spearman	-.381	0.352
OB Serum NT-proBNP at T0	OB Saliva NT-proBNP at T0	Spearman	0.213	0.397
OB Serum NT-proBNP at T4	OB Saliva NT-proBNP at T4	Spearman	0.260	0.917
HT Serum NT-proBNP at T0	HT Saliva NT-proBNP at T0	Spearman	-.119	0.638

HT Serum NT-proBNP at T4	HT Saliva NT-proBNP at T4	Spearman	-.091	0.720
OB+HT Serum NT-proBNP at T0	OB+HT Saliva NT-proBNP at T0	Spearman	0.056	0.746
OB+HT Serum NT-proBNP at T4	OB+HT Saliva NT-proBNP at T4	Spearman	-.013	0.938

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam; T1 = day of discharge for AHF patients; T4 = four week follow-up.*

Furthermore, we investigated the correlation of serum and saliva values of NT-proBNP only for AHF patients and only at T0, according to age (≤75 or >75 years old), sex (male or female), eGFR (30-44 mL/min, 45-59mL/min, >60mL/min), BMI (≤25, 25-20, >30), EF (≤40% or >40%) and presence of AF **(Table 17)**. There was a statistically significant correlation for serum and salivary NT-proBNP for AHF patients who are older than 75 years, and those who have atrial fibrillation **(Table 17)**; again groups of patients with higher biomarker values.

A statistically significant correlation for serum and salivary NT-proBNP was also found for various other AHF subgroups as described below: for those that did not receive RAAS blockers, those receiving beta blockers, those without LVH, those with body weight equal or less than 85 kilograms, those with a 6MWD ≤ 315m, WBC ≤ $8.4x10^3$, Hb ≤ 13.1d/dL, serum Creatinine ≥1.105mg/dL, Sodium≤135mEq/L, Glucose ≥124mg/dL, ferritin ≥23 mg/dL, TSH≤5.33 µU/mL, LVIDD>51.5mm, E>1.16m/sec wave, TRVmax ≥2.8m/sec and IVC insp ≤22mm **(Table 17)**. The value of these univariate correlations cannot be explored here and larger studies are needed to assess their potential clinical importance.

Table 17. Correlations of serum and salivary NT-proBNP in AHF sub-populations at timepoint 0.

Variable	Characteristic	Correlation Coefficient	P Value
Age, years	≤75	Spearman	0.102

	>75	Spearman	**0.047**
Sex	Male	Spearman	0.178
	Female	Spearman	0.176
eGFR mL/min/1.73m^2	30-44	Spearman	**0.07**
	45-59	Spearman	0.468
	>60	Spearman	0.875
BMI, kg/m^2	≤25	Spearman	0.088
	25-30	Spearman	0.221
	>30	Spearman	0.154
LVEF, %	≤40	Spearman	0.062
	>40	Spearman	0.111
AF	YES	Spearman	**0.002**
	NO	Spearman	0.376
NYHA	III	Spearman	0.295
	IV	Spearman	0.815
RAAS	NO	Spearman	**0.002**
B -BLOCKER	YES	Spearman	**0.005**
LVH	NO	Spearman	**0.046**
WEIGHT, kg	≤85	Spearman	**0.030**
6MWD, m	≤315	Spearman	**0.003**
WBC	≤8.4 X10^3	Spearman	**0.034**
Hb, g/dL	≤13.1	Spearman	**0.029**
Creatinine, mg/dL	>1.105	Spearman	**0.017**
Sodium, mEq/L	≤135	Spearman	**0.002**
Glucose, mg/dL	≥124	Spearman	**0.007**
Ferritin, ng/mL	≥23	Spearman	**0.030**
TSH, µU/mL	≤5.33	Spearman	**0.018**
LVIDD, mm	≥51.5	Spearman	**0.019**
E velocity, m/sec	>1.16	Spearman	**0.042**
TR Vmax, m/sec	≥2.8	Spearman	**0.007**
IVC inspiration, mm	≤21	Spearman	**0.032**

Abbreviations: 6MWD, six minute walk distance; AF, atrial fibrillation; BMI, body mass index; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IVC, inferior vena cava; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular

internal end diastolic diameter; NYHA, New York Heart Association; RAAS: renin-angiotensin-aldosterone system; TRVmax: tricuspid valve regurgitation velocity; TSH, thyroid-stimulating hormone; WBC,white lood cell count.

3.3 Investigation of non-linear correlation between serum and salivary NT-proBNP values.

For all study groups at timepoint 0, the correlation between serum and salivary NT-proBNP values had a correlation coefficient R less than 0.8, pointing that the relationship for the biomarker values of the two fluids is not linear. Therefore, we investigated a non linear correlation between serum and salivary NT-proBNP values for all study groups at T0. **Table 18** shows the model with the best coefficient of determination (R square) with and without a constant term. The salivary NT-proBNP was the independent variable and the serum NT-proBNP was the dependent variable.

For AHF patients (Group I), the best model with constant term is cubic (formula $Y=\beta_0+\beta_1X+\beta_2X^2+\beta_3X^3+\varepsilon$) with R square = 0.138, which explains the 13.8% of the distribution of Serum NT-proBNP for the whole study population at timepoint 0, according to the Saliva NT-proBNP values for the whole study population. The best model without constant term is power (equation is Y = b0 * (t**b1) or ln(Y) = ln(b0) + (b1 * ln(t))), with a R square=0.934, which explains the 93.4% of the distribution of Serum NT-proBNP for the whole study population values according to the Saliva NT-proBNP values for the whole study population.

For CHF patients (Group II), the best model with a constant term is cubic with R square = 0.603. The best model without constant term is power, with a R square=0.883, which means that this model explains the 88.3% of the distribution of Serum NT-proBNP for the whole study population at timepoint 0 according to the Saliva NT-proBNP values for the whole study population at timepoint 0.

For obese patients (Group III), the best model with and without constant term is cubic with R square = 0.842 and 0.876 respectively (84.2% and 87.6%).

For hypertensive patients (Group IV), the best model with constant term is cubic with R square = 0.16, which explains only the 16% of the distribution of Serum NT-proBNP for the whole study population values according to the Saliva NT-proBNP values for the whole study population at timepoint 0. The best model without constant term is power with a R square=0.867 which explains the 86.7% **(Table 18)**.

Table 18. Non linear correlation between serum and salivary NT-proBNP values at T0						
Group	**Model with constant term**	**R square**	**P value**	**Model without constant term**	**R square**	**P value**
I (AHF)	Cubic	0.138	0.08	Power	0.934	**0.001**
II (CHF)	Cubic	0.603	**0.051**	Power	0.883	**0.001**
III (obese)	Cubic	0.842	**0.001**	Cubic	0.876	**0.001**
IV (hypertensive)	Cubic	0.16	0.471	Power	0.867	**0.001**

*dependent variable=Serum NT-proBNP, independent variable= Salivary NT-proBNP

3.4 Multiple linear regression analysis of the factors affecting serum and salivary NT-proBNP values.

In section 3.2, we presented results derived from the use of simple linear models and correlations of NT-proBNP values measured both in serum and saliva samples. In section 3.3, we further explored non linear types of regressions between these two variables that led to interesting results; mathematical equations appeared to connect the saliva and serum values.

In this section, using multiple linear regression (MLR) techniques, we explore the existence of multiple linear relations, with serum NT-proBNP values for the total study population at timepoint 0 according to the Saliva NT-proBNP values for the whole study population at the same timepoint as dependent variables and all other variable [age, sex, history of AF, NYHA class, SBP, QRS duration, breathing rate, weight, BMI, 6MWD, medications (RAAS, beta blocker, MRA, diuretic), WBC, urea, sodium, eGFR, TBIL, LVEF, LVIDD, LAVI, E/E', TRVmax, IVC insp, EQ5D-5L scale] as independent predictors-explanatory variables (the terms "explanatory variables" and "predictors" are equivalent). Finally, we attempted a multiple linear regression analysis for determination of the factors affecting the serum and salivary NT-proBNP

values, and the identical factors that affect both. This study was carried out only for the AHF group.

In the first step we ran MLR models, with constant (intercept) and non-constant terms, and we included almost all other variables (numeric and dummy) as the independent explanatory variables. The available MLR methods, Forward, Backward, Stepwise, Remove (that are known to derive from these methods), were used in an attempt to select the best fitted models. In a second step, we conducted another set of MLR using selected explanatory variables (**Table 19**).

Among other questions, the key one we wanted to answer through this analysis was whether these two variables, namely Serum NT-proBNP at T0 and Saliva NT-proBNP at T0 have the same statistically significant explanatory variables for the AHF group, i.e. if their expected values can be predicted on the basis of same predictors.

Although the logical suggestion would be that the expected NT-proBNP values for saliva and serum can be predicted on the basis of same predictors, we see that the non-constant term models fit better for both variables under study. In the constant term and non-constant term models for serum, we have: R-Square=0,706 and 0,922 respectively, and for Saliva models we have R-Square =0,540 and 0,840. In the AHF group, the predictors for Serum NT-proBNP and Saliva NT-proBNP at T0 are NYHA, CHOL, weight, LVIDD, and urea, eGFR, and age respectively.

Therefore, these results indicate the saliva and serum NT-proBNP values cannot be predicted by the same parameters.

Table 19. Multiple Linear Regression in the Group of AHF. Predictors at baseline													
method	FORWARD All Variables Entered as explanatory												
Input variable	Serum NT-proBNP at baseline							Saliva NT-proBNP at baseline					
	model includes constant		VIF	model without constant				model includes constant			model without constant		
	Model	Values		Model	Values	VIF		Model	Values	VIF	Model	Values	VIF
	Constant	-5525,140						Constant	-39,990				
	NYHA	3356,043	1,074	NYHA	3285,411	24,612		Ur	0,365	1,816	Ur	,298	4,829
	CKD	3086,069	1,369	CHOL	-33,328	15,062		eGFR	0,651	1,816	eGFR	,448	8,264
	Diuretic	-2705,456	1,288	Weight	-55,135	16,702					Age	-,330	14,972
				LVIDD	62,124	21,901							
R		0,706			0,922				0,540[b]			0,849	
R-Square		0,499			0,849				0,291			0,721	
A R-Square		0,458			0,833				0,254.			0,699	
P-Value		**0,000**			**0,000**				**0,001**				
D-W		2,053			1,415				1,429			1,321	

Abbreviations: AF, atrial fibrillation; CHOL, cholesterol; D-W, CKD, chronic kidney disease; Durbin-Watson statistic; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVIDD, left ventricular internal end diastolic diameter; NYHA, New York Heart Association; QRS, complex duration in msec; RAAS: renin-angiotensin-aldosterone system; TRVmax: tricuspid valve regurgitation velocity; Ur, urea; VIF, variance inflation factor; WBC,white lood cell count.

3.5 Univariate correlations of serum and salivary NT-proBNP with various variables.

For the HF population (Groups I, II and combined Group I-II), significant univariate correlations of serum NT-proBNP levels with various studied parameters were observed, for time points 0, 1 (only for Group I), and 4. The studied parameters are shown in **Table 20.** Significant correlations are shown in **Table 21**, while the totality of the results are provided in **Table 21 extended (see Appendix)**.

Table 20. Variables assessed for univariate correlations with saliva and serum NT-proBNP

Somatometric parameters	Laboratory Investigations
Weight	White Blood Ceel Count (WBC)
Body Mass index (BMI)	Hemoglobin (Hb)
Vital signs	Urea
Systolic Blood Pressure (SBP)	Creatinine
Diastolic Blood Pressure (DBP)	Estimated Glomerular Filtration Rate (eGFR)
Heart Rate (HR)	Potassium (K+)
Breathing Rate (BR)	Sodium (Na+)
Saturation O2	Glucose (Glu)
Other Clinical Characteristics	Ferrum (Fe)
Presence of atrial fibrillation (AF)	Ferritin
QRS duration in ECG	Thyroid Stimulating Hormone (TSH)
NYHA functional class (I-IV)	Total Bilirubin (Tbil)
6-Min Walking Distance (6MWD)	Total Cholesterol (TCHOL)
EQ5D-5L score	LDL cholesterol (LDL-c)
Medications	Echocardiographic parameters
RAAS inhibitors / ARNI	Left Ventricular Ejection Fraction (LVEF)
MRA	Left Ventricle Internal End Diastolic Diameter (LVEDD)
Diuretics	Left Ventricular mass (LV mass)
Beta-Blockers	LV hypertrophy (LVH)
	E velocity, E/A ratio, E/e' ratio
	TR Vmax
	IVC exp, IVC insp

Regarding the CHF patients, the serum NT-proBNP at T0 was moderately positively associated with creatinine, and moderately inversely associated with estimated glomerular filtration rate (eGFR), and the score for quality of life in the EQ5D-LD questionnaire. At T4 (i.e. one month later) in CHF patients, the serum NT-proBNP was moderately positively associated with oxygen saturation ($SatO_2$) and ferritin and moderately inversely associated with eGFR.

For AHF patients, there were no extreme scores in the serum values, so asymptotic normality was assumed. Parametric and non parametric tests were performed, according to the normality tests and the percentage of extreme scores for the other variables. Serum NT-proBNP at T0 was moderately positively associated with urea, while it was also weakly positively associated with creatinine, inferior vena cavae diameter in expiration (IVC exp) and inspiration (IVC ins). It was also weakly inversely associated with systolic blood pressure (SBP), oxygen saturation ($SatO_{2)}$, the 6-minute walking distance (6MWD), and the quality of life EQ5D-5L score. Especially for NYHA functional class, only the NYHA III and NYHA IV classes were used, since no patients were NYHA I class and only two patients were NYHA II class. Thus, the Mann Whitney U test was used and statistically significant differences were found in median values; lower values in NYHA III vs NYHA IV [2919 (4663) vs 7470(3720) respectively, p=0.001].

On discharge from the hospital (Time-point 1), serum NT-proBNP for AHF patients was moderately inversely associated with diastolic blood pressure (DBP), total cholesterol (TCHOL), and low density cholesterol (LDL-chol), while a weak inverse association was found with hemoglobilin and positive association with urea. There was a statistically significant difference in median values of patients concerning the use of RAASi/ARNI; higher values for those receiving ACE or ARB)/ARNI compared to those who did not [median 4203(5362) vs 1456 (4668) respectively, p=0.028]. Regarding NYHA class and since there were only NYHA II and NYHA III patients, the Mann Whitney U test was used and we found statistically significant differences in median values; lower values in NYHA III vs NYHA IV [1412 (2557) vs 5359(6654) respectively, p=0.0001].

At one month follow-up (T4), serum NT-proBNP for AHF patients was moderately inversely associated with systolic blood pressure (SBP), eGFR, TCHOL, and LDL-chol, and moderately positively associated with breathing rate (BR), left atrial diameter (LA diam), E wave velocity of mitral diastolic flow, and E/E' ratio. A weak association was also found with urea, creatinine and total bilirubin (TBIL). Statistically significant differences in NT-proBNP values were found in relation to:

- the presence of LV hypertrophy (LVH); higher values in those with LVH vs those without LVH [median value 4130pg/mL (7565) vs 1208pg/mL (2114) respectively, Mann Whitney U test, p=0.049]

- the use of RAASi/ARNI use; lower values in those receiving RAASi/ARNI vs those not receiving [median value 1962pg/mL (2890) vs 5815pg/mL (6655 respectively), Mann Whitney U test, p=0.018],

- the diuretic use; lower values in those receiving diuretics vs those not receiving [median value 378pg/mL vs 3282pg/mL respectively, Mann Whitney U test, p=0.036] and

- the NYHA class. As there were 3 classes (NYHA I-III), the Kruskal Wallis test for independent samples was used and revealed that there was statistically significant differences in the serum NT-proBNP values between NYHA class II and IV; lower values in NYHA II vs NYHA IV [2128pg/mL (3530) vs 8546pg/mL (4396) respectively, p=0.023].

For the combined populations of AHF and CHF patients (Groups I and II), at T0 there was a moderate positive correlation of serum NT-proBNP with urea, creatinine, and IVC at inspiration, and a moderate inverse association with 6MWD, TCHOL. There was a weak positive correlation with TBIL, E/E' ratio, and tricuspid valve regurgitation velocity (TR Vmax). There was also a weak inverse correlation with SBP, $SatO_2$, eGFR, LDL chol, and EQ5D-5L score. Regarding NYHA class, 3 classes (NYHA II-IV) were taken into account (because of only two patients with NYHA class I), so the Kruskal Wallis test for independent samples was used: statistically significant differences were found in the serum NT-proBNP values between NYHA class II and

NYHA IV [median NT-proBNP values 3835pg/mL (1924) vs 7470pg/mL (3720) respectively, (p=0.001)], and between NYHA class III and IV [1652pg/mL (4595) vs 7470pg/mL (3720) respectively, (p=0.002)].

At T4, the serum NT-proBNP for the combined populations of AHF and CHF patients (Groups I and II) had a moderate positive correlation with urea, LA diam, E wave velocity, E/E' ratio, TR Vmax, a moderate inverse correlation with SBP, eGFR, TCHOL, LDL chol, EQ5D-5L score, and weak correlations for the other variables of Table 12 (like the QRS duration). There were statistically significant differences in NT-proBNP values in relation to:

1) presence of LVH; median value 4130pg/mL (7565) for those with LVH vs 1208pg/mL (2114) for those without LVH (Mann Whitney U test, p=0.049)

2) the use of RAASi/ARNI; median value 1899pg/mL (2696) for those receiving RAASi/ARNI vs 4562pg/mL (6845) for those not receiving, (Mann Whitney U test, p=0.041)

3) the diuretic use; median value 378pg/mL for those on diuretics vs 2404pg/mL fort jose not receiving diuretics (Mann Whitney U test, p=0.006].

4) the NYHA class. As there were 3 classes (NYHA I-III), the Kruskal Wallis test for independent samples was used and revealed that there was statistically significant differences in the serum NT-proBNP values between NYHA class II and IV [median values 1962pg/mL (3122) vs 8546pg/mL (4396) respectively, p=0.016).

Table 21. Significant (p<0.05) Univariate correlations of serum NT-proBNP with clinical, laboratory and echocardiographic parameters

CHF patients at T0, n=12	Variable	Correlation Coefficient	R value	P value
	Creatinine	Spearman	0.592	**0.043**

	eGFR	Spearman	-0.664	**0.018**
	EQ5D-5L	Spearman	-0.664	**0.044**
CHF patients at T4, n=8	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	$SatO_2$	Spearman	0.727	**0.041**
	WBC	Spearman	0.714	**0.047**
	Ferritin	Spearman	0.775	**0.041**
	eGFR	Spearman	-0.976	**0.0001**
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.298	**0.037**
	Urea	Pearson	0.408	**0.004**
	$SatO_2$	Spearman	-0.339	**0.017**
	Creatinine	Pearson	0.385	**0.006**
	IVC exp	Spearman	0.378	**0.007**
	IVC insp	Pearson	0.363	**0.010**
	EQ5D-5L	Pearson	-0.327	**0.022**
	6MWD	Pearson	-0.399	**0.005**
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.294	**0.043**
	EQ5D-5L	Pearson	-0.287	**0.048**
	RAASi/ARNI use	Mann Whitney		**0.028**
	DBP	Pearson	-0.434	**0.002**
	TCHOL	Spearman	-0.69	**0.001**
	LDL-chol	Spearman	-0.618	**0.001**
	Hb	Pearson	-0.301	**0.038**
	Urea	Pearson	0.319	**0.027**
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.474	**0.002**

	LVH yes	Mann Whitney		**0.049**
	RAAS use	Mann Whitney		**0.018**
	Diuretic use	Mann Whitney		**0.036**
	BR	Spearman	0.401	**0.009**
	eGFR	Pearson	-0.428	**0.005**
	TCHOL	Spearman	-0.591	**0.0001**
	LDL-chol	Spearman	-0.522	**0.001**
	LA diam	Spearman	0.503	**0.039**
	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	Urea	Pearson	0.362	**0.019**
	Creatinine	Pearson	0.325	**0.035**
	TBIL	Pearson	0.352	**0.024**
AHF and CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	6MWD	Pearson	-0.411	**0.001**
	SBP	Pearson	-0.258	**0.045**
	IVC exp	Pearson	0.425	**0.001**
	Urea	Pearson	0.487	**0.0001**
	Creatinine	Pearson	0487	**0.0001**
	TCHOL	Pearson	-0.4	**0.001**
	IVC insp	Pearson	0.446	**0.0001**
	$SatO_2$	Spearman	-0.358	**0.005**
	eGFR	Pearson	-0.396	**0.002**
	TBIL	Pearson	0.302	**0.019**
	LDL-chol	Pearson	-0.361	**0.004**
	E/E' ratio	Pearson	0.263	**0.048**
	TR Vmax	Pearson	0.265	**0.039**
	EQ5D-5L	Pearson	-0.395	**0.002**
AHF and CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.43	**0.002**
	Urea	Pearson	0.471	**0.001**
	eGFR	Pearson	-0.524	**0.0001**

	TCHOL	Pearson	-0.542	**0.0001**
	LDL-chol	Pearson	-0454	**0.002**
	LA diam	Spearman	0.503	**0.039**
	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	TR Vmax	Spearman	0.446	**0.037**
	EQ5D-5L	Pearson	-0.408	**0.003**
	QRS	Pearson	0.280	**0.049**
	BR	Pearson	0.384	**0.006**
	Creatinine	Pearson	0.455	**0.001**
	LVH yes	Mann Whitney		**0.049**
	RAASi/ARNI use	Mann Whitney		**0.041**
	Diuretic use	Mann Whitney		**0.006**

Abbreviations: 6MWD, six minute walk distance; ARNI, angiotensin receptor-neprilysin inhibitor; BR, breathing rate; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IVC exp, inferior vena cava diameter at expiration; IVC insp, inferior vena cava diameter at inspiration; LAdiam, left atrium diameter; LDL-chol, low-density lipoprotein; LVH, left ventricular hypertrophy; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; SBP, systolic blood pressure; TBIL, total bilirubin; TCHOL, total cholesterol; TRVmax, tricuspid regurgitation maximum velocity; WBC, white blood cell count.

For the HF population (Groups I, II and combined Group I-II) significant correlations of salivary NT-proBNP levels with various studied parameters were found, for time points 0, 1 (only for Group I), and 4. The studied parameters are shown in **Table 22**. Significant correlations are shown in **Table 22**, while the totality of the results are provided in **Table 22 extended (see Appendix)**.

For salivary values in CHF patients, non parametric tests showed that the saliva NT-proBNP at T0 was moderately positively associated with QRS duration, body weight, and LV mass, and negatively associated with heart rate and EQ5D-5L score. At T4 for CHF patients, non parametric tests showed that the salivary NT-proBNP was moderately associated only with body weight.

For salivary NT-proBNP values in AHF patients, parametric and non parametric tests showed that the saliva NT-proBNP at T0 had weak correlations with the variables studied, with a tendency for positive correlation with creatinine, TBIL, urea, and a moderate positive correlation for E/A ratio. At T1 there was only a moderate correlation with MRA use and a weak inverse association with heart rate and EQ5D-5L score; the median (IQR) NT-proBNP values were 9.6pg/mL (18.06) for those receiving MRA and 19.16pg/mL (17.71) for those not receiving MRA [Mann Whitney U test, p=0.045]. At T4, there was statistically significant difference for diuretic use; median value was 13.33pg/mL for those receiving diuretics vs 49.82pg/mL for those not on diuretics (Mann Whitney U test, p=0.048]. There was also a moderate correlation with BMI and IVC diameter in expiration.

The salivary NT-proBNP of the combined AHF and CHF patients (all HF population, Groups I and II) at T0 was only weakly positively correlated with weight, LV mass, breathing rate, urea, creatinine, TBIL, E/A ratio, and IVC on expiration, and weakly inversely correlated with 6MWD. At T4, for the combined Group I and II population there was statistically significant difference in the salivary NT-proBNP according to diuretic use; median value 13.24 (24.61)pg/mL for those on diuretics vs 49.82pg/mL for those not receiving diuretics (Paired Samples T test, p=0.01]. There was a moderate positive correlation with weight, BMI, IVC diameter in expiration and a moderate inverse correlation with oxygen saturation.

Table 22.Significant (p<0.05) and near significant (p<0.1) Univariate correlations of salivary NT-proBNP with clinical, laboratory and echocardiographic parameters.

CHF patients at T0, n=12	Variable	Correlation Coefficient	R value	P value
	QRS	Spearman	0.605	**0.037**
	Weight	Spearman	0.636	**0.026**
	LV mass	Spearman	0.7	**0.036**
	HR	Spearman	-0.62	**0.032**
	EQ5D-5L	Spearman	-0.523	0.081

CHF patients at T4, n=8	Variable	Correlation Coefficient	R value	P value
	Weight	Spearman	0.735	**0.038**
	RAASi/ARNI use	Levene's		0.071
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Creatinine	Spearman	0.294	**0.040**
	TBIL	Spearman	0.298	**0.038**
	E/A ratio	Spearman	0.468	**0.021**
	Urea	Spearman	0.247	0.087
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	HR	Pearson	-0.323	**0.027**
	6MWD	Pearson	-0.255	0.088
	MRA use	Mann Whitney		**0.045**
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	BMI	Pearson	0.371	**0.016**
	IVC exp	Spearman	0.404	0.062
	Diuretic use	Mann Whitney		**0.048**
AHF+CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Spearman	0.248	0.054
	LV mass	Spearman	0.236	0.075
	BR	Spearman	0.230	0.075
	6MWD	Spearman	-0.276	**0.033**
	Urea	Spearman	0.233	0.071
	Creatinine	Spearman	0.345	**0.006**
	TBIL	Spearman	0.252	0.052
	E/A ratio	Spearman	0.378	**0.027**
	IVC exp	Spearman	0.273	**0.033**

	EQ5D-5L	Spearman	-0.250	0.052
AHF+CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Pearson	0.306	**0.031**
	$SatO_2$	Spearman	-0.309	**0.029**
	BMI	Pearson	0.392	**0.005**
	IVC exp	Spearman	0.404	0.062

Abbreviations: 6MWD, six minute walk distance; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; BR, breathing rate; HR, heart rate; IVC exp, inferior vena cava diameter at expiration; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; TBIL, total bilirubin.

3.6 Serum and salivary Cortisol comparisons among study groups.

Regarding the serum and saliva Cortisol values, we compared all groups (AHF, CHF, obese and hypertensive) at timepoint T0; we also compared the different groups at the same timepoint, similar to what we did for NT-proBNP. When tests of normality failed, non parametric test were performed. The comparisons are shown in **Table 23**.

For serum cortisol, there were differences only at T0 (baseline) for AHF patients when compared to all other groups, and for combined HF populations compared to non HF patients; levels were higher in AHF vs all other groups and in HF population compared to the non-HF population. No statistically significant differences were found for salivary cortisol values among groups and timepoints (data not shown).

Table 23. Comparison between Serum Cortisol values among study groups at timepoint T0

Variable 1	Median (IQR)	Variable 2	Median (IQR)	P value, Mann Whitney test
AHF	15.30 (7.65)	CHF	6.99 (8.70)	**0.018**
AHF	15.30 (7.65)	Obese	9.05 (4.27)	**0.0001**

AHF	15.30 (7.65)	Hypertensive	10.20 (5.05)	**0.003**
AHF	15.30 (7.65)	Non-HF (obese and hypertensive)	9.60 (4.5)	**0.0001**
HF population (AHF+CHF)	14.45 (7.67)	Non-HF (obese and hypertensive)	9.60 (4.5)	**0.001**
CHF	6.99 (8.70)	Obese	9.05 (4.27)	1.000
CHF	6.99 (8.70)	Hypertensive	10.20 (5.05)	0.669
CHF	6.99 (8.70)	Non-HF (obese and hypertensive)	9.60 (4.5)	0.845

AHF = acute HF; CHF= chronic HF; T0=day of admission or day of first exam

Concerning the correlations among serum and salivary cortisol levels, tests were performed for all study groups and at timepoint T0. A statistically significant correlation between salivary and serum cortisol was observed only at baseline (T0) for the non-HF population, i.e the combined obese and hypertensive patients **(Table 24)**.

Table 24. Correlation between Serum and Salivary Cortisol levels at baseline (T0)

Population	**Correlation Coefficient**	**R value**	**P value**
All patients, n=97	Pearson	0.206	0.100
AHF patients, n=49	Spearman	0.202	0.293
CHF patients, n=12	Spearman	-0.036	0.939
Obese, n=18	Pearson	0.462	0.072
Hypertensives, n=18	Spearman	0.516	0.071
Non-HF population (Obese and Hypertensive), n=36	Spearman	**0.471**	**0.010**

HF population (AHF+CHF), n=61	Spearman	0.144	0.402

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam.*

3.7 Correlation between Serum and salivary NT-proBNP and Cortisol levels in HF patients.

The correlation between the two biomarkers in both serum and saliva was assessed in the total population, and all HF groups (AHF, CHF, combined AHF+CHF) at all timepoints (T0, i.e. baseline for all, T1, i.e at discharge only for AHF patients, T4, i.e. at 4 weeks for all). When tests of normality failed, non parametric test were performed. Statistically significant correlations were found only among serum bio markers and at most time points for the whole as well as the HF population, while no significant correlations were found between the salivary biomarkers at any timepoint **(Table 25)**. Serum NT-proBNP was moderately correlated with serum cortisol for the whole study population both at baseline (R value 0.502) and at 4 weeks (R value 0.540); in AHF patients, serum NT-proBNP was moderately correlated with serum cortisol at T4 (R value 0.433). In CHF patients, serum NT-proBNP was strongly correlated with serum cortisol at T0 (R value 0.821). Finally, for combined AHF and CHF patients, serum NT-proBNP was moderately correlated with serum cortisol both at T0 (R value 0.454) and T4 (R value 0.453).

Table 25. Correlation between NT-proBNP and Cortisol levels in serum and in saliva.

Serum NT-proBNP and Serum Cortisol	Correlation Coefficient	R value	P value
All patients at T0, n=97	Spearman	**0.502**	**0.0001**
All patients at T4, n=86	Spearman	**0.540**	**0.0001**
AHF patients at T0, n=49	Spearman	0.315	0.096
AHF patients at T1, n=49	Spearman	0.261	0.171

AHF patients at T4, n=42	Spearman	**0.433**	**0.009**
CHF patients at T0, n=12	Spearman	**0.821**	**0.023**
CHF patients at T4, n=8	Spearman	0.393	0.383
HF population (AHF+CHF) at T0, n=61	Spearman	**0.454**	**0.005**
HF population (AHF+CHF) at T4, n=50	Pearson	**0.453**	**0.003**
Saliva NT-proBNP and Saliva Cortisol	**Correlation Coefficient**	**R value**	**P value**
All patients at T0, n=97	Spearman	-0.080	0.438
All patients at T4, n=86	Spearman	0.114	0.296
AHF patients at T0, n=49	Spearman	-0.075	0.611
AHF patients at T1, n=49	Pearson	0.190	0.202
AHF patients at T4, n=42	Pearson	-0.032	0.840
CHF patients at T0, n=12	Spearman	-0.483	0.112
CHF patients at T4, n=8	Spearman	0.143	0.736
HF population (AHF+CHF) at T0, n=61	Spearman	-0.214	0.098
HF population (AHF+CHF) at T4, n=50	Pearson	0.013	0.929

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam; T1 = day of discharge for AHF patients; T4=four week follow-up.*

4. DISCUSSION

In this clinical study, we investigated the role of salivary biomarkers (primarily NT-proBNP and secondarily cortisol) in the management (diagnosis, stratification and assessment of response to treatment) of HF patients, in both the acute and chronic setting. These biomarkers were also measured in serum, especially since NT-proBNP is considered to be the gold standard biomarker for HF diagnosis; NT-proBNP values <125pg/mL make the diagnosis of HF unlikely [4], while for acute HF the threshold is 300pg/mL [4]. As many factors are known to affect NT-proBNP serum levels, e.g. age, presence of AF, impaired renal function, and obesity [495-496], these were also taken into account in this study.

Four groups of patients were studied; AHF (on admission, discharge and at 1 month after discharge), CHF, obese and hypertensive patients who were not diagnosed with HF (at baseline and 1 month after the first visit). Importantly, the 4 groups had some differences that will be discussed further below.

The main findings of the study are:

A. **<u>Comparison of baseline NT-proBNP levels among the 4 groups</u>**

A statistically significant difference in the serum NT-proBNP levels between obese and hypertensive patients, with lower values found in obese compared to hypertensive patients, although a worse lipid profile, greater LV dimensions, a lower regional wall thickness and higher E/A ratio were found in obese compared to hypertensive individuals. Furthermore, hypertensive patients received more anti-hypertensive medications, while the lowest utilization rate of medications was found in the obese group. However, it should be noted that obesity is known to be associated with lower than expected NT-proBNP values [65, 66] and thus, this difference between the 2 groups could be attributed to obesity. Regarding the saliva levels, no significant difference was found between these 2 groups, indicating that this saliva biomarker may not be useful to differentiate individuals with low biomarker values.

Serum values of NT-proBNP in the combined HF population (all HF patients) were significantly higher when compared to non-HF patients, both at baseline and at 1

month. Importantly, the HF population (AHF and CHF) were older compared to non HF-patients; although aging is associated with higher NT-proBNP levels, the difference in NT-proBNP levels in our groups cannot be solely explained by the difference in age. The salivary NT-proBNP levels in the combined HF population (all HF patients) were significantly higher compared to the non-HF patients (obese and hypertensive), both at baseline and at 1 month, as expected. This result comes in agreement with previous studies [395] where salivary NT-proBNP differed among HF and healthy subjects.

Serum NT-proBNP levels were significantly higher in CHF patients compared to non-HF patients both at baseline and at 1 month. CHF patients also had a higher prevalence of CAD, AF and impaired renal function, while they received more anti-HF medications. CHF patients (and hypertensives) were significantly older compared to obese patients. As expected, CHF patients had a larger LV, lower LVEF, higher LV filling pressures (as indicated by the E/E' ratio) and estimated systolic pulmonary artery pressures (as indicated by the TR maximal velocity) and walked a lower distance in 6MWT compared to the non-HF population. In contrast to the serum biomarker, the salivary NT-proBNP levels of CHF patients showed no significant difference compared to any other group.

Serum NT-proBNP levels in AHF patients were significantly higher when compared to CHF, or any other study group, both at baseline and at 1 month after discharge. This may be attributed to increased congestion in AHF patients as indicated by higher NYHA class, lower 6MWD values, increased E/E' and inferior vena cavae diameter in AHF compared to CHF patients. Of note, no significant differences were found in age, gender, cardiovascular disease history, or medications between AHF and CHF patients. Serum NT-proBNP values decreased significantly from admission to discharge for the AHF patients, confirming previous studies in AHF [497], while there was no statistically significant difference from discharge to 1 month.

However, the salivary NT-proBNP levels in AHF patients were significantly higher when compared to hypertensives or the non-HF patients (obese and hypertensive), both at baseline and at 1 month, as expected. However, no significant difference was found when compared to CHF patients, at baseline or any time point. This finding probably indicates that although salivary NT-proBNP may differentiate between HF

and nonHF populations, it does not appear to have a value in differentiating between AHF and CHF. The lack of difference between AHF at baseline and obese individuals is difficult to explain and may likely be due to small sample size.

B. **Relation of serum and saliva NT-proBNP levels in various groups of patients.**

The saliva values of the biomarker were significantly lower compared to the serum values in all cases; this, taken into account their large variability, probably indicates that larger samples (especially regarding the saliva measurements) would be needed to show any significant difference with an intervention.

A significant linear correlation, albeit weak, between serum and salivary NT-proBNP was found only in the following cases: 1) for the whole study population at baseline, 2) for the combined HF population at baseline and 3) for the AHF group at baseline and at discharge. Importantly, In these cases in which a significant linear correlatin was observed, samples were either relatively large or the biomarker values were high. This is probably why a significant correlation was found between serum and salivary NT-proBNP levels for AHF patients who were older than 75 years, and those who had AF; both groups had higher NT-proBNP values, as expected [495-496]. This may be hypothesized in other AHF subgroups associated with higher biomarker levels (and worse prognosis), e.g. those who did not receive RAAS blockers, those receiving beta blockers, those with lower body weight, lower 6MWD, lower sodium, higher LV filling pressures and pulmonary artery pressure.

The poor correlation between biomarker levels in saliva and serum may suggest an impaired transportation of NT-proBNP from the blood circulation into the saliva; a less efficient mechanism of converting the precursor molecule of proBNP, by furin convertase, into NT-proBNP, since its enzymatic activity in saliva is inhibited by histatins, preventing the in situ generation of salivary NT-proBNP, has been previously suggested [498-499]. The correlation of serum and salivary NT-proBNP values in AHF patients shown in this study [500], is also supported by Foo [400]. However, the 200:1 ratio for serum and saliva values, reported by Foo et al [400], does not appear to apply in our study. On the contrary, Dekker et al found no correlation between serum and salivary BNP in AHF patients [394]. The association

between saliva and serum levels of NT-proBNP in AHF patients (both at baseline and at discharge) may suggest that saliva NT-proBNP could potentially be used for monitoring of treatment effects in patients with AHF (in-hospital or early post-discharge), using serial measurements.

Although the linear correlation for serum and salivary NT-proBNP is weak, when we investigated a non-linear correlation for all study groups at timepoint 0 (with the salivary NT-proBNP as the independent variable and the serum NT-proBNP as dependent variable), a much better correlation was found, reaching R square values of 0.88-0.93, which explains this 88-93% of the distribution of serum levels according to the saliva NT-proBNP values. Even for the obese and hypertensive patients, a satisfactory non linear correlation was found, with R square values of 0.87-0.88, that could explain the 87-88% of the corresponding distribution. These results indicate that a relationship of the salivary NT-proBNP with the established serum biomarker exists, but appears to be non-linear and more complex. Further researh is needed to further explore this.

Multiple linear regression techniques with serum and saliva NT-proBNP values as dependent variables and all other variables (demographic, clinical, laboratory and echocardiographic) as potential independent predictors showed that the dependent variables do not have the same predictors. This interesting finding could be explained by the disturbances of enzymatic and non-enzymatic antioxidant defense, and the oxidative damage to proteins and lipids that take place in the saliva with the progression of HF. Furthermore, a secretory dysfunction of salivary glands in patients with HF compared to age- and gender-matched control group may also contribute [389, 501].

C. **Stability in serum and saliva NT-proBNP from baseline to 1 month in stable patients.**

The serum NT-proBNP levels did not differ between baseline and 1 month in any of the stable patient groups (CHF, obese or hypertensives), indicating a satisfactory repeatability of the measurements, although this was not formally evaluated in our study. Similarly, the saliva NT-proBNP levels did not differ between baseline and 1

month in any of the groups. Whether this indicates good repeatability or lack of statistical power cannot be resolved here.

D. **Association of the serum and saliva NT-proBNP levels with clinical characteristics.**

The serum NT-proBNP levels appear to be associated with different parameters in the CHF and AHF groups. In most cases, these associations are only moderate and as they refer to rather small groups, a causal association cannot be established. Some of the associations regarding the serum NT-proBNP levels that may be considered important are presented below:

- CHF patients.

  Serum NT-proBNP at baseline was inversely associated with eGFR and QoL score using the EQ5D-LD questionnaire.

  A multivariate regression analysis was not performed because of a very small number of subjects.

- AHF patients.

  Serum NT-proBNP at baseline was associated with urea, creatinine, IVC diameter and the NYHA class and inversely associated with SBP, $SatO_2$, 6MWD and the QoL score.

  In multivariate regression analysis, independent predictors were identified to be: NYHA class, LV internal diameter (both positive association), weight and total cholesterol (both negative association) in one analysis and in another: NYHA, diagnosis of CKD (positive) and use of diuretics (negative association).

  Serum NT-proBNP at discharge from the hospital was associated with urea, NYHA class and use of RAAS/ARNI and inversely associated with SBP, DBP, total and LDL cholesterol, hemoglobilin and the QoL score.

  Serum NT-proBNP at 1 month was associated with NYHA class, presence of LVH, left atrial diameter, E wave velocity, E/E' ratio, breathing rate, urea, creatinine and total bilirubin and inversely associated with SBP, eGFR, total and LDL cholesterol, the use of RAAS/ARNI and diuretics.

  All these associations, at all time points (baseline, at discharge and 1 month) may be attributed to disease severity.

- Combined HF population (CHF and AHF patients).

  Serum NT-proBNP at baseline was associated with creatinine, urea, total bilirubin, E/E' ratio, IVC diameter, TR maximal velocity, and NYHA class, and inversely associated with SBP, 6MWD, total and LDL cholesterol, satO2, eGFR and the QoL score.

  Serum NT-proBNP at 1 month was associated with urea, left atrial diameter, E wave velocity, E/E' ratio, TR maximal velocity, QRS duration, breathing rate, NYHA class, presence of LVH, the use of RAASi/ARNI and diuretics. An inverse correlation was observed with SBP, eGFR, total and LDL cholesterol, the QoL EQ5D-5L score.

  All these associations at baseline and 1 month may be attributed to disease severity.

The salivary NT-proBNP levels were also associated with different parameters in the CHF and AHF groups. In most cases, these associations were only weak to moderate (i.e. worse compared to serum) and as they refer to rather small groups, a causal association cannot be established. Furthermore, the pathophysiological explanation of these associations may be hard to describe. Some of the associations regarding the salivary NT-proBNP levels that may be considered important are presented below.

- CHF patients.

  Salivary NT-proBNP at baseline was associated with QRS duration, body weight, and LV mass, and inversely associated with heart rate and EQ5D-5L score.

  Salivary NT-proBNP at 1 month was associated only with body weight, a finding that may be due to concealed congestion.

- AHF patients.

  Saliva NT-proBNP at baseline was significantly (albeit weakly) correlated with some the variables studied, i.e. creatinine, total bilirubin, and E/A ratio. In multivariate regression analysis, independent predictors were identified to be: urea and eGFR (both positive association), a finding difficult to explain because of the complex pathophysiology of AHF.

Saliva NT-proBNP at discharge was correlated with MRA use and inversely associated with heart rate; this may have been attributed to increased congestion or advanced disease leading to more medications' use.

Saliva NT-proBNP at 1 month was associated with body weight and diuretic use, probably indicating higher congestion.

Looking at the findings regarding serum and salivary NT-proBNP correlations with clinical characteristics in the total HF population, only the serum correlations do confirm expected correlations (based on the pathophysiology and clinical presentation of HF). In contrast to serum, salivary biomarker correlations cannot be easily explained and indicate the weakness of the biomarker measured in saliva.

E. **Change in serum and saliva NT-proBNP levels with treatment in AHF patients.**

A significant decrease in serum NT-proBNP was noted between baseline and discharge in AHF patients, but not between discharge and 1 month follow-up. Regarding saliva levels in AHF, a numerical decrease was noted from baseline to discharge, but this was not foud to be significant. Given the association between saliva and serum levels of NT-proBNP in AHF patients (both at baseline and at discharge) in our study, it may be concluded that the saliva biomarker could be considered as a promising marker for monitoring AHF patients and assessing the effect of interventions, but it is not currently a reliable marker. More research is needed to potentially improve this.

F. **Main findings regarding cortisol levels**

Serum cortisol levels were significantly higher in AHF vs all other groups and in the HF population compared to the non-HF population. No statistically significant differences were found for salivary cortisol values among groups and time points (data not shown). As salivary cortisol did not appear to have any meaningful difference among groups, its change over time and its potential correlation with other clinical parameters was not further studied.

A statistically significant correlation between salivary and serum cortisol was observed only at baseline for the non-HF population (combined obese and hypertensive patients).

G. **Correlation between NT-proBNP and cortisol levels**

A significant correlation were found only between those two biomarkers (NT-proBNP and cortisol) when these were measured in serum. This was a consistent finding for most time points (although their association was weak or moderate in most cases), for the whole population as well as the combined HF population, probably indicating that the two serum biomarkers both reflect disease severity or myocardial stress. In contrast, no significant correlation was found between the salivary biomarkers at any timepoint. Given the totality of our data, it might be primarily attributed to problems associated with cortisol measurement in the saliva.

Comment on the 4 patient groups.

Our HF population consists of HF patients with various causes of HF and from the whole range of LVEF spectrum. According to the study design, HF patients were matched to nonHF controls (obese and hypertensives) based on the presence of similar symptoms, e.g. dyspnoea on exertion. HF patients were older compared to controls, mostly males, and with a greater prevalence of cardiovascular disease (i.e. CAD, strokes or transient ischemic attacks, PAD, AF etc). Matching to age was not attempted as it is very difficult to find 'healthy' individuals of the age range 60-75years. This may explain some of the differences observed between the HF and nonHF groups, i.e. in saliva levels and clinical parameters (such as the 6MWD).

Individuals in the obese group had a greater prevalence of smoking and a lower prevalence of a history of the other traditional CVD risk factors (hypertension, dyslipidemia, diabetes). However, when their blood samples were tested and abnormally raised total cholesterol and low density lipoprotein levels were found, a diagnosis of dyslipidemia was made. Obese and hypertensive subjects were not included as a single combined control group as originally planned, because of the fact that the serum NT-proBNP values were significantly different between these two patient groups and thus this was not considered to be 'statistically' appropriate. However, it is known that obesity lowers natriuretic peptide values [502], and perhaps, from a 'clinical' point of view, it would be reasonable to unite those non-HF patients to a single control group.

To our knowledge, our study was the first clinical study that compared saliva and serum NT-proBNP in these patient groups (non-HF controls, CHF, and AHF). A previous study investigated the association between serum and saliva BNP levels in hospitalized HF patients and found no association between saliva and serum [394 Dekker]. In that study, patients had a functional capacity of NYHA stages I-III, with 50% of them classified as NYHA I-II [394], while our study included patients with worse NYHA classes [61% of CHF patients were NYHA class III and 98% of AHF patients were NYHA class III-IV, and 38% of them NYHA IV]. Another study measured salivary BNP and serum NT-proBNP in controls, CHF, and AHF patients [395]. In that study, saliva BNP levels were shown to be higher in HF populations (CHF and AHF) compared with non-HF patients while a significant correlation between salivary BNP and plasma NT-proBNP concentration was also reported [395]. In total, the results of this study are largely similar to our study. The third study that measured salivary NPs in CHF patients (NYHA III, reduced LVEF <40%) reported significantly higher saliva NT-proBNP levels in CHF patients compared to healthy controls [400]. The discrepancy in results between our study and previously published ones, may be attributed to differences in the populations studied, the type of NP measured (BNP vs NT-proBNP), the method of NPs measurement in blood but mainly in saliva, as well as the sample storage conditions. Of note, a common finding in all studies concerning salivary NPs (including the current study) was the very low concentration of NPs in saliva [394, 395, 400].

5. CONCLUSIONS

In this clinical study, the role of salivary biomarkers (primarily NT-proBNP and secondarily cortisol) in the management (diagnosis, stratification and assessment of response to treatment) of HF patients, in both the acute and chronic setting, was investigated. Our HF population consisted of HF patients with various causes of HF and from the whole range of LVEF spectrum. According to the study design, HF patients were matched to nonHF controls (obese and hypertensives) based on the presence of similar symptoms (dyspnea on exertion).

In the non-HF groups, a statistically significant difference was found in the serum NT-proBNP levels between obese and hypertensive patients, with lower values found in obese compared to hypertensive patients; thus, the non-HF patients were studied as 2 different groups. Regarding the saliva levels, no significant difference was found between these 2 groups.

The saliva values of the biomarkers were significantly lower compared to the serum values in all cases. A significant linear correlation between serum and salivary NT-proBNP was found only in the following cases: for the whole study population at baseline, for the combined HF population at baseline and for the AHF group at baseline and at discharge, but all correlations were moderate to weak. Beyond the linear correlations, a non-linear and more complex relationship of the salivary NT-proBNP with the established serum biomarker was shown; further research is needed for this to be described in detail. The serum NT-proBNP levels did not differ between baseline and 1 month in any of the stable patient groups. Similarly, the saliva NT-proBNP levels did not differ between baseline and 1 month in any of the groups. Whether this is due to satisfactory repeatability in biomarker measurement should be a matter of future research.

Serum values of NT-proBNP in the combined HF population (all HF patients) were significantly higher compared to non-HF patients (obese and hypertensive combined), both at baseline and at 1 month. Similarly, salivary NT-proBNP levels in the combined HF population were significantly higher compared to the non-HF patients, both at baseline and at 1 month.

Serum NT-proBNP levels were significantly higher in CHF patients compared to non-HF patients both at baseline and at 1 month. In contrast, the salivary NT-proBNP levels of CHF patients showed no significant difference compared to any other group.

Serum NT-proBNP levels in AHF patients were significantly higher when compared to CHF, or any other study group, both at baseline and at 1 month after discharge. The salivary NT-proBNP levels in AHF patients were significantly higher when compared to hypertensives or the combined non-HF patients. No significant difference was found when compared to either obese alone or CHF patients, at any time point.

Taken all these into account, serum NT-proBNP can differentiate between HF and non-HF groups as well as between AHF and CHF; this makes it an established biomarker in HF diagnosis. On the contrary, salivary NT-proBNP could probably be used only in diagnosing AHF patients from a general non-HF population.

In AHF, serum NT-proBNP levels decrease significantly from baseline to discharge, while saliva levels show a numerical decrease was noted from baseline to discharge, which was not foud to be significant. Given an association is shown between saliva and serum levels of NT-proBNP in AHF patients (both at baseline and at discharge) in our study, it may be concluded that the saliva biomarker could be considered only as a promising marker for monitoring AHF patients and assessing the effect of interventions, in need for more research.

Looking at the findings regarding serum and salivary NT-proBNP correlations with clinical characteristics in HF patients, only the serum levels' correlations confirm expected associations (based on the pathophysiology and clinical presentation of HF). Thus, all serum NT-proBNP associations in AHF patients and the combined HF population at all time points (baseline, at discharge and 1 month) may be attributed to disease severity. In contrast, the salivary NT-proBNP levels were associated with different parameters in the CHF and AHF groups. These salivary biomarker associations were only weak to moderate (and certainly worse compared to the serum ones) and as they refer to rather small groups, they cannot establish a causal association. However, potential pathophysiological explanations may apply; enzymatic and non-enzymatic saliva processes and salivary gland dysfunction during

HF progression have been previously suggested to contribute to these differences between saliva and serum measurements.

Regarding serum cortisol, levels were significantly higher in AHF vs all other groups and in the HF compared to the non-HF population. No statistically significant differences were found in salivary cortisol values among groups and time points. Lastly, a significant correlation was found only between NT-proBNP and cortisol, when measured in serum, at baseline and at 1 month. In contrast, no significant correlation was found between the salivary biomarkers at any timepoint. Looking at the study data as a whole, this might be primarily attributed to the pitfalls or technical problems associated with cortisol measurement in the saliva.

LIMITATIONS

The main limitation of this study were the unavoidable consequences of the SARS-CoV-2 pandemic, which started having an impact in Europe by February 2020 and had serious implications in this particular protocol that required saliva samples. On one hand, stable chronic HF patients (suitable for enrollment in Group II) and stable non-HF patients (suitable for enrollment in Group III and IV) were worldwide encouraged to avoid scheduled appointments in the Outpatients Departments, in order to release sources for the pandemic, while at the same time the Outpatients' Departments had periodically cancelled their services because of the SARS-CoV-2 pandemic. This situation caused large difficulties in the enrollment of patients for this study. In addition, some of the patients who had agreed to participate and gave their consent, were found to be positive for SARS-CoV-2 and their samples had to be destroyed after being collected, when the results of the PCR test became available. A small percentage of acute HF patients were unwilling to return to the hospital for their follow-up, especially when pandemic was getting periodically worse. As the study had just started when the pandemic occurred, as part of a larger international study, these risks were foreseen and the study protocol was modified. However, fewer than originally predicted patients were enrolled in the study and groups of patients studied were rather small for this type of clinical study. Furthermore, and as this study was part of a larger protocol that secured the funding for the measurement of the biomarkers (KardiaTool funded by the Horizon2020 initiative), there was no realistic option to increase the sample size, especially during the pandemic.

On the other hand, the total population size (approximately 100 subjects) may be considered to be satisfactory and compares well with most of the clinical trials that used saliva samples for biomarkers' assessment [394-395, 400]. As expected with small sample size, in many cases, the data did not meet the normality criteria and and non-parametric tests had to be used. This may have led to incorrect inferences in small samples such as the groups II-IV in our study. Due to the small size of the groups, ANOVA could not be used for comparison among all groups and unpaired comparisons were performed increasing the systematic error in the analysis.

The 4 patient groups had differences in their baseline characteristics; these may partially explain ther differences observed when groups were compared. Similarly, beyond the univariate correlations, a multivariate analysis was performed to assess independent associations of biomarkers with clinical or other characteristics, but did not show any statistically significant results. Further research with larger sample sizes is needed to evaluate this.

The reagent kit used for serum NT-proBNP measurements is based on biotinylated polyclonal anti-NT-proBNP antibodies and gold-labeled monoclonal anti-NT-proBNP antibodies, while for the saliva NT-proBNP measurement, the kit uses AA 32–57 (polyclonal sheep anti-human NT-proBNP) as capture antibody and AA 8–29 (HRP-labeled polyclonal sheep anti-human NT-proBNP) as detection antibody. This difference in the methodology may have had an important impact on the correlation of serum and saliva levels of this biomarker.

The reproducibility of the measurements of the biomarkers in saliva was not formally assessed. As this was a preliminary evaluation of these saliva biomarkers, a typical reproducibility study that would allow for a Blland-Altman analysis, was not performed. However, measurements done with a 4-week interval in the stable patients' groups (CHF, obese and hypertensive) showed similar values with no statistical differences.

Some hypertensive patients from the control group were prescribed RAAS inhibitors (i.e. ACE inhibitors and angiotensin receptor blockers), MRAs, or beta-blockers for the treatment of their hypertension; this may have limited the difference in biomarkers between the HF and non-HF population. However, no sifnificant correlations between medications' use and biomarkers was found.

Since our study began before the 2021 ESC guidelines for the management of HF were published, many of our patients did not receive sodium–glucose cotransporter 2 inhibitors (SGLT2i); a new study with HF patients (especially AHF patients) who receive all four pillars of HF might show more robust results. However, it should be noted that a common side effect of SGLT2i is xerostomia, which could further

decrease saliva production and make salivary measurements of any biomarkers more challenging.

The samples were stored at -20^{o} C, since previously reported studies have shown that although cortisol is more stable and can last up to 4 weeks at 4 °C [503], the optimal storage conditions for salivary NT-proBNP were at -20°C and can be preserved for up to 2.5 months [504].

Finally, this study does not allow to draw any conclusions on the role of salivary biomarkers in prognosis. Apart from the small number of patients, no follow-up beyond the 4 weeks was performed because of the COVID-19 pandemic.

6. FUTURE RESEARCH

As measuring biomarkers in saliva appears to be very convenient for patients, avoiding thus blood sampling, methods to improve saliva excretion in HF patients need to be explored, while methods to measure biomarker levels in an accurate and reproducible way in saliva should be improved. The variability of these measurements according to food and fluid intake and time of the day should be assessed as well as their relation to gender, age, body mass index, eGFR, use of diuretics.

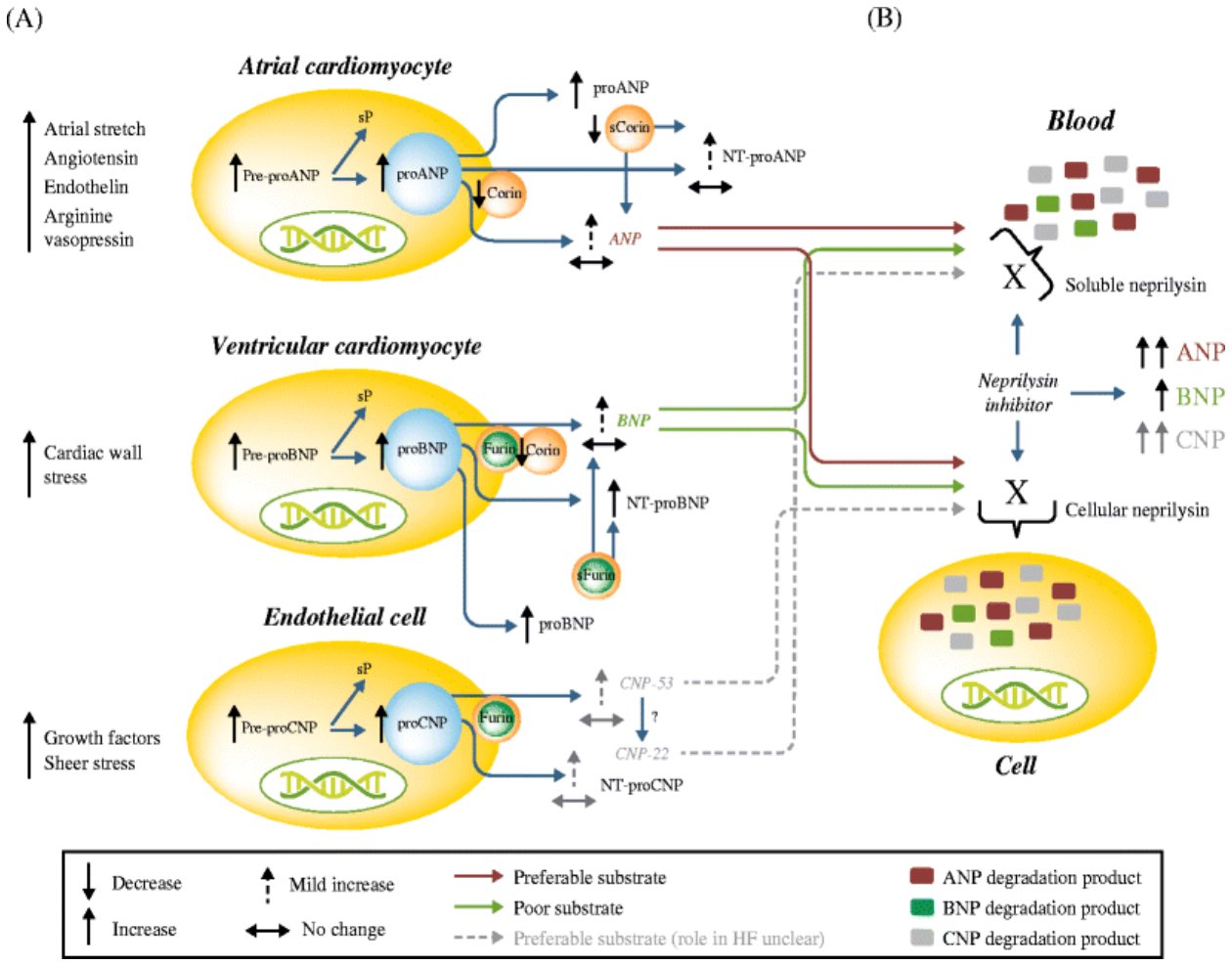
The diagnostic role of salivary NT-proBNP may be more important in AHF patients as indicated by the salivary NT-proBNP levels that were found to be significantly higher in AHF patients compared to non-HF groups and the significant correlation between serum and salivary NT-proBNP in subgroups of AHF (older than 75 years or with atrial fibrillation, i.e. patient groups in whom NT-proBNP values are known to be higher). Larger studies in these populations might show a more important role for salivary NT-proBNP in the diagnosis of AHF.

Furthermore, the role of saliva NT-proBNP in the assessment of treatment response in AHF patients should be further explored in larger studies. Salivary NT-proBNP levels decreased, albeit non-significantly, from baseline admission to discharge in AHF patients, reflecting some response to treatment. The moderate negative association of salivary NT-proBNP with MRA and diuretic use, not in the acute phase, but on discharge and after one month, i.e. at the stabilization after an acute decompensation, also indicates a potential value in monitoring response to treatment. These two categories of medications are fundamendal in the treatment of congestive HF and if a stronger correlation could be proved, salivary NT-proBNP might have a place in treatment modifications and follow-up of patients with a recent episode of acute HF on a chronic setting. Although the number of HF patients we used is comparable or larger than previously reported studies with saliva biomarkers in HF, larger populations and a longer follow-up would be needed to make stronger results.

Saliva also contains other molecules, such as electrolytes (sodium, potassium, etc.) that are extremely important in the management of HF patients [404, 505]. Future studies are needed to evaluate the feasibility of theris measurement and their usability in a clinical setting. A potential combination of these biomarkers with NT-proBNP might be useful for monitoring HF therapy and management guidance.

APPENDIX

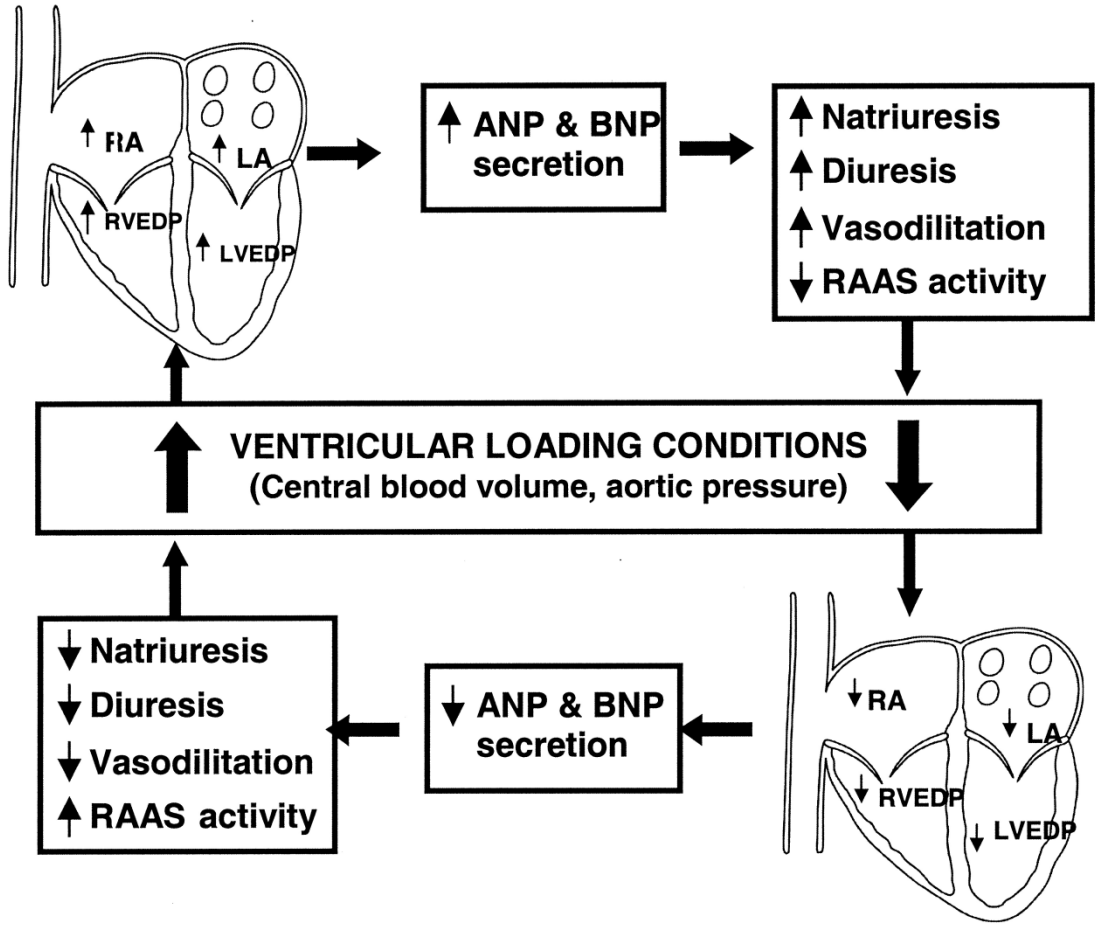
Figure 1. Synthesis and processing of NPs via neprilysin in patients with HF.



Synthesis and processing of NPs via neprilysin in patients with HF **a)** ANP is synthesized in atrial cardiomyocytes as pre-proANP; the sP of which is cleaved to form proANP. Upon secretion, proANP is processed by membrane-bound and soluble corin, generating an N-terminal peptide (NTproANP; 98 amino acids), and an active C-terminal peptide (ANP; 28 amino acids). In patients with HF, corin levels are decreased, resulting in an increase in predominantly unprocessed ANP. BNP is synthesized as pre-proBNP in ventricular cardiomyocytes. Removal of a sP from pre-proBNP forms proBNP, which is processed by membrane-bound and soluble furin, and corin, to release the N-terminal portion (NT-proBNP; 76 amino acids), and the biologically active C-terminal (BNP; 32 amino acids). CNP is widely expressed in the vasculature and found in high concentrations in the endothelium. CNP expression has also been reported in cardiomyocytes at gene and protein levels. CNP is

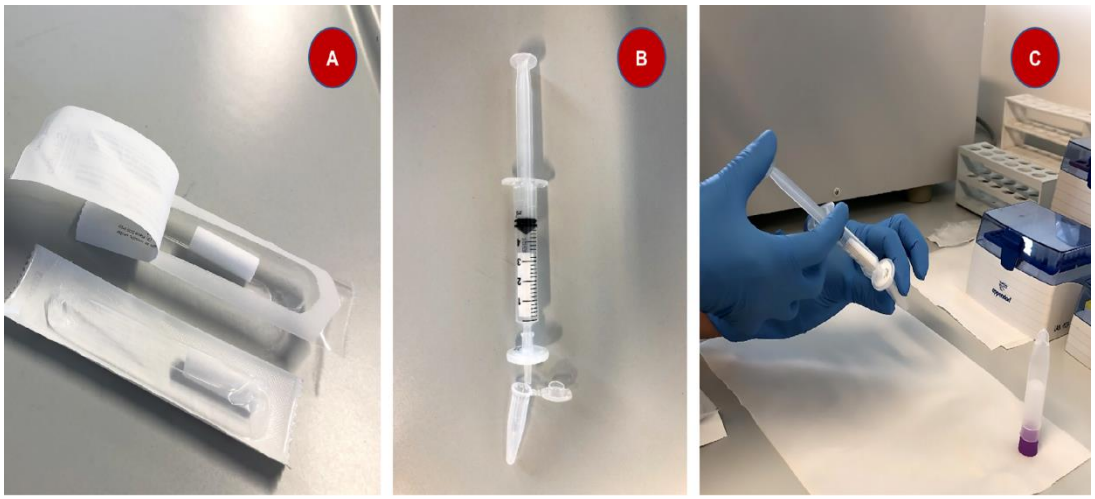
synthesized as pre-proCNP; the sP of which is cleaved to form proCNP. Processing of proCNP (103 amino acids) may occur via furin to yield a53 amino acid C-terminal peptide (CNP-53), the major active form of CNP in the tissues. In the systemic circulation, a 22 amino acid form of CNP dominates (CNP-22), but the protease responsible for this cleavage is unknown. **b)** Cellular and circulating soluble neprilysin are major contributors to ANP degradation. In contrast, BNP is a poor neprilysin substrate. Thus, neprilysin inhibition (e.g., via sacubitril) is most likely associated with greater augmentation of ANP activity than BNP activity. Neprilysin also has a high affinity for CNP, and as such, inhibition of neprilysin is expected to increase levels of CNP. *ANP* atrial natriuretic peptide, *BNP* B-type natriuretic peptide, *CNP* C-type natriuretic peptide, *HF* heart failure, *NP* natriuretic peptide, *NTproANP* N-terminal proANP, *NT-proBNP* N-terminal proBNP, *NTproCNP* N-terminal proCNP, *sP* signal peptide, *TGF* transforming growth factor (*from Rubattu and Tryposkiadis 2017*).

Figure 2. Mechanism by which cardiac secretion of natriuretic peptides serves to maintain intravascular volume homeostasis.



Schematic diagram depicting the mechanism by which natriuretic peptide secretion serves to maintain intravascular volume homeostasis. ANP = atrial natriuretic peptide; BNP = B-type natriuretic peptide; LA = left atrium; RA = right atrium; LVEDP = left ventricular end-diastolic pressure; RAAS = renin-angiotensin-aldosterone system; RVEDP = right ventricular end-diastolic pressure.

Figure 3. Oral swab procedure for saliva collection.



An oral swab (a) was placed in the space between the buccal wall and the oral gingival or beneath the tongue, for two minutes. The swab was then collected with sterile gloves and placed in a syringe, at the tip of which a filter was placed to remove any content (b). The saliva sample was infused in a plastic tube (c) to be centrifuged at 4000rpm for two minutes, before storage at -20°C.

Table 1: Etiology of Heart Failure	
Myocardial Diseases	
Ischemic heart disease	
Toxic damage	Recreational substance use, heavy metals, medications, radiation
Infectious diseases	Bacteria, spirochaetes, fungi, protozoa, parasites (Chagas disease), rickettsiae, viruses (HIV/AIDS).
Inflammatory - autoimmune diseases	Lymphocytic/giant cell myocarditis, Graves' disease, rheumatoid arthritis, connective tissue diseases, eosinophilic myocarditis (Churg–Strauss).
Infiltrative diseases	Amyloidosis, sarcoidosis, haemochromatosis, glycogen storage diseases, lysosomal storage diseases.
Malignancies	Primary or metastatic
Genetic cardiomyopathies	Hypertrophic, dilated, non-compaction, arrhythmiogenic, restrictive, muscular dystrophies, laminopathies.
Metabolic diseases	Thyroid and parathyroid diseases, hypercortisolemia, Conn's disease, Addison disease, diabetes, metabolic syndrome, phaeochromocytoma, pathologies related to pregnancy and peripartum.
Nutritional disorders	Deficiency of thiamine, L-carnitine, selenium, iron, phosphates, calcium.
Abnormal Loading Conditions	
Hypertension	
Valvular diseases	
Structural defects	Atrial and ventricular septum defects
Pericardial diseases	Constrictive pericarditis, pericardial effusion
Endocardial diseases	Hypereosinophilic syndrome, endomyocardial fibrosis, endocardial fibroelastosis
Volume overload	Renal failure, self-induced water intoxication, iatrogenic

	fluid overload
High output conditions	Anemia, sepsis, thyrotoxicosis, arteriovenous fistula, pregnancy, Paget's disease.
Arrhythmias	
Bradyarrhytmias	Sinus node dysfunction, conduction disorders
Tachyarrhytmias	Ventricular, atrial arrhytmias

Table 2. Serum biomarkers that have shown a potential role in HF management		
Biomarker	**Clinical Usefulness**	**References (No)**
Natriuretic peptides	Diagnosis	(1), (4), (59-60)
	Prognosis	(68-96)
	Therapy monitoring	(97-108)
Troponin	Assists in diagnosis	(4), (60)
	Prognosis	(116-141)
Gal-3	Diagnosis	(60), (153)
	Prognosis	(143-156)
	Therapy monitoring	(158)
ST2	Diagnosis	(163)
	Prognosis	(159-168),(171)
	Therapy monitoring	(169-170), (466)
FGF-23	Prognosis	(173-177), (179)
Copeptin	Prognosis	(185-194)
GDF-15	Diagnosis	(209-210), (212)
	Prognosis	(201-208), (213)
ET-1	Diagnosis	(216)
	Prognosis	(217-221), (223-231)
CRP	Prognosis	(234-248)
IGFBP-7	Prognosis	(249-250), (254-256), (258-259)
	Potential Diagnosis	(117)
Albumin	Prognosis	(260-267)
Ca-125	Prognosis	(269-275), (280-282), (285-286)
	Therapy monitoring	(463-465)
UA	Prognosis	(293-299)
MR-proADM	Prognosis	(310-317)
	Diagnosis (in AF)	(319)
	Therapy monitoring	(466)

Cystatin C	Prognosis	(324-334), (337-340)
CCN1/CYR61	Prognosis	(341-344)
Endotrophin	Therapy monitoring	(345)
Non coding RNAs (circ and micro)	Diagnosis	(117), (346), (353)
	Prognosis	(352-353)
	Therapy targets	(346)
MMPs	Support diagnosis	(117), (356), (359)
	Prognosis	(356-360), (362)
Tetranectin	Prognosis	(374)
Neprilysin	Diagnosis in RD	(379)
	Prognosis	(377)
Pentraxin 3	Prognosis	(381)
vWF	Prognosis	(383)
HDL-P	Prognosis	(384)
Apolipoprotein M	Prognosis	(385)
Soluble CD14	Prognosis	(386)
GlycA	Prognosis	(387)
LRG1	Prognosis	(388)
Chloride	Prognosis	(363-365), (368-371)

RD=Renal dialysis patients

Table 3. Saliva biomarkers that have shown a potential role in HF management (3).					
Author [Reference]	**Year**	**Saliva Biomarker**	**Range (in Total Study Population)**	**Correlation between Saliva and Serum Levels**	**Clinical Usefulness**
Foo et al (398).	2012	NT-proBNP	18.3–748.7 pg/mL	No correlation	Diagnosis
Joharimoghadam et al (393).	2017	BNP	4.96–6.85 ng/L	Not assessed	Diagnosis Monitoring
Denver et al (401).	2000	Endothelin	0.50–23.56 fmol/mL	r = 0.536, p = 0.032	Diagnosis Monitoring
Zhang et al (396).	2016	Gal-3	2.8–2510 ng/mL	r = 0.4, p < 0.01	Diagnosis
Zhong et al (408).	2019	Gal-3	16.04–869.3 ng/mL	Not assessed	Prognosis (association with cardiovascular death or hospitalization)
Zhang et al (394).	2017	S10A7	Not assessed	Not assessed	Diagnosis
Hammer et al (395).	2016	Cortisol	0.08–1.28 ng/mL	Not assessed	Prognosis (association with mortality)
Alhurani et al (397).	2014	Cortisol	0.09–0.55 µg/dL	Not assessed	Prognosis (association with cardiac event free-survival)

Jekell et al (400).	2004	Cortisol	15–23 nmol/L	Not assessed	Monitoring
Klimiuk et al (389).	2020	Uric Acid	0.5818–0.862 ng/mL	Not assessed	Diagnosis, Monitoring
Ghimenti et al (390).	2020	8-isoPGF$_{2\alpha}$	25–60 pg/mL	Not assessed	Monitoring
Wolfram et al (399).	2005	8-epiPGF2α	43–111 pg/mL	Not assessed	Diagnosis, Monitoring
Ghimenti et al (390).	2020	Lactate	190–3790 mmol/L	Not assessed	Monitoring
Klimiuk et al (389).	2020	Amylase	0.12–0.21 µmol/mg	Not assessed	Diagnosis (lower levels in HF)
White et al (402).	1950	Sodium, Chloride, Potassium	7.4–27.9 12.8–30.8 16.2–29.7 mEq/L	No correlation found	Monitoring

Table 4. Urine biomarkers that have shown a potential role in HF management					
Author [Reference]	**Year**	**Urinary Biomarker**	**Range (in Total Study Population)**	**Correlation between Urine and Serum Levels**	**Clinical Usefulness**
Ng et al (421).	2005	NT-proBNP	0.54-1.103.8 fmol/mL	Not mentioned	Diagnosis
Cortes et al (420).	2007	NT-proBNP	60-120pg/mL	1:9-1:15	Monitoring
Comnick and Ishani (423).	2011	Albumin	N/A	N/A	Prognosis
DeBoer et al (427).	2018	Albumin Creatinine Ratio	N/A	N/A	Prognosis
Maisel et al (433).	2011	NGAL	60-200ng/mL	N/A	Prognosis
Nakada et al (430).	2017	NGAL	N/A	N/A	Prognosis
Labr et al (432).	2018	NGAL	N/A	N/A	Prognosis
Verbrugge et al (436).	2013	IL-18	10.97-46.56 pg/mL	N/A	Prognosis (AKI in ADHF)
Damman et al (438).	2010	KIM-1	188-537) ng/g creatinine	N/A	Prognosis
Damman et al (438).	2010	NAG	6–17 U/g creatinine	N/A	Prognosis

Jungbauer et al (439).	2011	KIM-1	620-1920 ng/g creatinine	N/A	Prognosis, Monitoring
Jungbauer et al (439).	2011	NAG	6-22 U/g creatinine	N/A	Prognosis
Zakeri et al (440).	2013	CNP22,CNP53	N/A	CNP22 limited association (p=0.28, p=0.04) CNP53 no relationship	Prognosis
Brinkley et al (441).	2018	Sodium	134-141 mmol/L	N/A	Prognosis (30 day)
Hou et al (443).	2014	ORM1	1.033.02-13.244,76 ng/mg	N/A	Diagnosis
Zhang et al (446).	2021	microRNA-423-5p	N/A	N/A	Prognosis (180 day)
Zhang et al (447).	2017	CIB1	0.65-8.23 µg/L	N/A	Diagnosis
Steckl (448).	2018	Cortisol	0.03-0.3 µM	N/A	Diagnosis
Campbell et al (445).	2020	Proteomic classifier HF1	0.30-2.46	N/A	Diagnosis

Table 5.Clinical trials using natriuretic peptides for biomarker-guided therapy							
Study	**N**	**Population**	**Timepoints**	**Primary end point**	**Follow up duration (median)**	**Improved Outcome**	**Ref**
Troughton et al, 2000	69	NYHA II-IV, HFrEF	Every 3 months	Composite of CVD, HFH, HF decompensation	9.5 months	Yes	(453)
STARS-BNP, 2007	220	NYHA II-III, LVEF<45%, on OMT	Every month for 3 months, then every 3 months	CVD or HFH	15 months	Yes	(454)
TIME-CHF, 2009	499	NYHA II-IV, LVEF≤45%	1,3,6,12,18 months after discharge	18-month survival free of hospitalizations and QoL	18 months	No	(455)
Anguita et al, 2010	60	NYHA III-IV,	NA	Composite of all-cause mortality and HFH	16±4 months	No	(456)
PRIMA, 2010	345	AHF	Discharge, 2weeks,1,3,6,9,12, 15,18,21,24 months	Survival without HFH	24 months	No, but advanced detection of HF events	(457)
SIGNAL-HF, 2010	252	NYHA II-IV, LVEF<50%	Baseline, <10days, 1,3,6,9 months	Composite of days alive, days out of hospital, and KCCQ score	9 months	No	(458)
BATTLESCARRED, 2010	364	AHF	Every 3 months	All-cause mortality	36 months	No, but improves long-term mortality in ≤75years old	(459)
Berger et al, 2010	278	NYHA III-IV, HFrEF, AHF	1,3,6,12 months after discharge	All-cause mortality, HFH	12 months	Yes	(460)
STARBRITE, 2011	130	NYHA III-IV, LVEF≤35%, AHF	1 week, 1,2,3,4 months after discharge	Survival without HFH	3 months	No	(461)
UPSTEP, 2011	279	NYHA III-IV,	Week 2,6,10,16,	Composite of all-cause death,	12 months	No	(462)

		LVEF<40%, AHF in past month	24,36,48, then every 6 months	hospitalization and diuretic up-titration			
PROTECT, 2010	151	NYHA II-IV, LVEF≤40%, AHF event	Every 3 months	Composite of worsening HF, HFH, clinically significant VA, ACS, cerebral ischemia, and CVD	6 months	Yes	(463)
NORTHSTAR, 2013	407	NYHA I-II (85%), LVEF≤45%	Every 1-3months	Composite of all-cause mortality and HFH	30 months	No	(464)
HABIT, 2013	167	NYHA I-IV, AHF, 70% HFrEF 30% HFpEF	Every day	ADHF events: CV death, ADHF admission or iv therapy or changes in oral HF medications	60 days after discharge	Yes	(100)
GUIDE-IT, 2017	110	LVEF<40%, high risk patients for HF events	At 2 weeks and every 3 months	Composite of CVD, HFH	15 months	No	(104)
TIME-CHF, 2017	499	NYHA II-IV, LVEF≤45%	1,3,6,12,18 months after discharge	Recurrent HFH and all-cause hospitalizations and/or all-cause death	5 years follow-up	Yes only for <75 years; improved HF hospitalization-free survival	(105)
PRIMA II, 2018	405	ADHF with NT-proBNP >1700 ng/L	Not predefined	Composite of all-cause mortality and HFH in 180 days, and number of days alive out of the hospital in 180 days	6 months	No; in biomarker arm discharge NT-proBNP more reduced	(465)

ACS: acute coronary syndrome, ADHF: acutely decompensated heart failure, CVD: cardiovascular death, HFH: heart failure hospitalization, KCCQ: Kansas City Cardiomyopathy Questionnaire, NA: Not applicable, OMT: optimal medical treatment, QoL: quality of life, VA: ventricular arrhythmia.

Table 6: Data Collection		
Parameter	**Units**	**Range**
General demographic variables (baseline collection only)		
Age	Years	>18
Gender	Male/Female	
Study Group	I/II/III/IV	
Diagnosis of HF	Yes/No	
If HF diagnosis Yes Year of diagnosis LVEF at time of diagnosis Aetiology Implantable Device	 Year % Ischemic HF/Hypertensive HF/Dilated Cardiomyopathy/ Post-chemotherapy/ Valvular/Other Yes/no	 1980-2021 10-80 Ischemic HF/Hypertensive HF/Dilated cardiomyopathy/ Post-chemotherapy/ Valvular/Other Yes/No
Personal history of cardiovascular disease (baseline collection only)		
History of MI	Yes/No	
CAD confirmed by angiography only	Yes/No	
History of CVA/TIA	Yes/No	
PAD	Yes/No	
PAF/AF	Yes/No	
VHD	Yes/No	
Classical cardiovascular risk factors (baseline collection only)		
Hypertension; consistent increase in BP (SBP ≥140mmHg +/- DBP ≥90mmHg) or >1 month use of antihypertensive medications.	Yes/No	
Hypercholesterolemia; defined as CHOL >200mg/dl (5.0 mmol/L) and/or LDL	Yes/No	

>115mg/dl (3.0 mmol/L) or receiving lipid-lowering therapy		
DM; defined as fasting blood glucose >126mg/dl (7mmol/L) or glycaeted haemoglobin >6.5% or use of antidiabetic medications	Yes/No	
Other chronic medical conditions (baseline collection only)		
Obesity; defined as body mass index (calculated as weight in kilograms divided by height in meters squared) >30 kg/m^2	Yes/No	
CKD; defined as eGFR (MDRD or EPI) <60ml/min/1.73m^2 for 3 months and/or overt proteinuria (albumin >300mg/g in spot morning sample)	Yes/No Mild or moderate CKD (eGFR 45-60 or 30-45) If yes, specify (relevant for clinicians only)	
Autoimmune disease	Yes/No	
Pulmonary disease	Yes/No	
Endocrine disease	Yes/No	
Malignancies	Yes/No	
Other	Yes/No	
Family History (baseline collection only)		
of CVD	Yes/No	
of DM	Yes/No	
of CMP	Yes/No	
Lifestyle / Habits (baseline collection only)		

Smoking	Never smoked /Ex (>1 year)/Current smoker	
Alcohol	Never/Mild (<1 units/day)/Moderate (1/day female, 2 units/day male)/High intake	
Physical exercise	Never/Sedentary/Moderate (as per guidelines)/ Athlete	
Allergies		
Allergies	Yes/No	
Symptoms		
NYHA class	I,II, III, IV	
Dyspnoea	Yes/No	
Orthopnoea	Yes/No	
Paroxysmal nocturnal dyspnoea	Yes/No	
Reduced exercise tolerance	Yes/No	
Cough	Yes/No	
Chest pain on exertion	Yes/No	
Other symptoms	Describe	
Physical Examination		
SBP	mmHg Arm blood pressure will be collected using an automated blood pressure measurement method after sitting for 5 minutes	70-200
DBP	mmHg Arm blood pressure will be collected using an automated blood pressure measurement method after sitting for 5 minutes	40-120
ECG	Sinus rhythm/Atrial fibrillation/flutter QRS duration, msec A resting 12 lead electrocardiogram should be	60-200

	performed	
Heart rate	Beats per minute (bpm) *Heart rate will be recorded from the resting ECG*	40-150
Respiratory rate	Breaths per minute	5-35
Oxygen saturation	% *Oxygen saturation will be measured using a pulse oximeter of a peripheral digit or an earlobe*	80-100
Height	cm	130-200
Weight	kg	40-150
BMI	kg/m^2	17-50
Murmurs	MR / TR / AS ?none	
Crackles	No / only base / above base	
S3 sound	Yes / No	
Oedemas	No / mild / moderate	
Jugular distention	cm	0-20
Hepatomegaly	cm	0-20
Cachexia	Yes/No	
Other findings	Describe	
Exercise tolerance (provided that the patient's clinical condition allows for)		
Six-minute walk test	Distance covered, meters HR response, bpm SBP response, mmHg DBP response, mmHg SatO2 response, %	0-700 40-200 70-200 40-150 70-100
Medications (category and whether they are on maximal tolerated dose apart from loop diuretics, for which total dose in mg should be noted)		
ACE inhibitor	Yes/No, maximal/submaximal	
ARB	Yes/No, maximal/submaximal	
ARNI	Yes/No, maximal/submaximal	
Beta blocker	Yes/No, maximal/submaximal	
MRA	Yes/No, maximal/submaximal	
Loop diuretic	Yes/No	

	Dose total mg	0-500
Thiazide diuretic	Yes/No	
Ivabradine	Yes/No	
Antiplatelet	Yes/No	
Anticoagulant	Yes/No	
Statin	Yes/No	
Calcium Channel Blocker	Yes/No	
Amiodarone	Yes/No	
Digoxin	Yes/No	
Alpha blockers	Yes/No	
Other medications	Yes/No Please specify (relevant for clinicians only)	
Vaccinations		
Influenza vaccine in last 12 months	Yes/No/Unknown	
Pneumococcal vaccine ever	Yes/No/Unknown	
Laboratory findings		
Full Blood Count including Hb WBC	 g/dl /µl	 7.0-20.0 2000-25000
NT-proBNP	pg/ml	0-50000
Urea	mg/dl	10-350
Creatinine	mg/dl	0.4-5.0
Sodium	mmol/L	100-180
Potassium	mmol/L	2.00-8.00
eGFR	ml/min/1.73 m^2	0-150
AST	IU/l	0-300
ALT	IU/l	0-300
TBL DBL	mg/dl mg/dl	0.4-9.0 0-9.0
CHOL	mg/dl (CF 0.0259 to mmol/L)	50-400
HDL	mg/dl (CF 0.0259 to mmol/L)	0-100
LDL	mg/dl (CF 0.0259 to mmol/L)	20-300

TRG	mg/dl (CF 0.0113 to mmol/L)	0-500
Fasting glucose	mg/dl (CF 0.0555 to mmol/L)	40-450
Fe FERR (baseline only)	µg/dl ng/ml	0-500 0-1000
TSH (baseline only)	µIU/ml	0-20
Echocardiogram		
LV Ejection fraction	%	10-80
LVEDD	mm	30-90
IVSd	mm	5-30
PWd	mm	5-30
RWT		0-1
LVmass	g	50-400
LVH	Yes/No	
Left atrial size	LA diameter (PLAX), mm LAVI (average 4ch & 2ch), ml/m^2	20-80 10-150
E	cm/s	0-250
E/A ratio		0-5
E/E' average		0-50
TR Vmax	cm/s	0-500
Inferior vena cava (IVC)	Rest, mm Inspiration, mm	0-50 0-50
Significant valvulopathies	Yes/No	
MR grade	1, 2, 3, 4	
MS grade	1, 2, 3, 4	
AS grade	1, 2, 3, 4	
AR grade	1, 2, 3, 4	
TR grade	1, 2, 3, 4	
Clinical information at Follow up visit		
Any hospital admissions since last visit?	Yes/No	
If Yes please categorise reason	HF related / CV related non HF / Non CV related	

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; (P)AF, (paroxysmal)atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CHOL, total cholesterol; CKD, chronic kidney disease; CMP,

cardiomyopathy; CVA, cardiovascular accident; DBP, diastolic blood pressure; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; FERR, ferritin; HF, heart failure; IVSd, interventricular septum thickness at end-diastole; LAVI, left atrial volume index; LDL, low-density lipoprotein; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVEDD, left ventricular end diastolic diameter; MI, myocardial infarction; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; PWd, Posterior wall thickness at end-diastole; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack; TRG, triglycerides; TRVmax, tricuspid regurgitation maximum velocity; TSH, thyroid-stimulating hormone; VHD, valvular heart disease; WBC, white blood cell count.

Table 7. The EQ5D-5L QUESTIONNAIRE	
MOBILITY	
I have no problems in walking about	
I have slight problems in walking about	
I have moderate problems in walking about	
I have severe problems in walking about	
I am unable to walk about	
SELF-CARE	
I have no problems washing or dressing myself	
I have slight problems washing or dressing myself	
I have moderate problems washing or dressing myself	
I have severe problems washing or dressing myself	
I am unable to wash or dress myself	
USUAL ACTIVITIES (e.g. work, study, housework, family or leisure activities)	
I have no problems doing my usual activities	
I have slight problems doing my usual activities	
I have moderate problems doing my usual activities	
I have severe problems doing my usual activities	
I am unable to do my usual activities	
PAIN/DISCOMFORT	
I have no pain or discomfort	
I have slight pain or discomfort	
I have moderate pain or discomfort	
I have severe pain or discomfort	
I have extreme pain or discomfort	

ANXIETY/DEPRESSION	
I am not anxious or depressed	
I am slightly anxious or depressed	
I am moderately anxious or depressed	
I am severely anxious or depressed	
I am extremely anxious or depressed	

SCALE 0-100:

Table 8: Overview of Study Evaluations in Group I

Activity	Follow-up time point		
	Day 0 (= T0)	Discharge (=T1)	Week 4 after discharge (=T4)
Log of suspected cases	X		
Eligibility assessment	X		
Selection (if necessary)	X		
Enrolment/Consenting	X		
Blood	X	X	X
Saliva	X	X	X
Demographic, clinical history, medication and risk factor assessment	X		X
MACE or hospitalisation since last visit			X
Physical examination	X	X	X
Vital signs at rest	X	X	X
ECG	X	X	X
Echocardiogram	X		X
6MWT	X if the patient could perform	X	X

Table 9. Overview of Study Evaluations in Group II, III and IV		
Activity	**Follow-up time point**	
	Day 0 (=T0)	Week 4 (=T4)
Log of invited cases	X	
Eligibility assessment	X	
Selection (if necessary)	X	
Enrolment/Consenting	X	
Specimen Collection		
Blood	X	X
Saliva	X	X
Clinical variables		
Demographic, clinical history, medication, and risk factor assessment	X	X
Major adverse cardiovascular event or hospitalisation since last visit		X
Physical examination	X	X
Vital signs at rest	X	X
ECG	X	X
Echocardiogram	X	Unless clinical situation changed within previous 1 month.
6MWT	X	X

Table 10. Descriptive statistics at baseline (time point 0)				
	Acute HF, n=49 Group I	**Chronic HF, n=12 Group II**	**Obese, n=18 Group III**	**Hypertensive, n=18 Group IV**
Age, years*	72±12	72±9	50±11	65±10
Male gender, n (%)	35 (70)	7 (54)	8 (44)	9 (50)
CV risk factors, n (%)				
Hypertension	43 (86)	10 (77)	5 (28)	18 (100)
Dyslipidemia	38 (76)	11 (85)	7 (39)	15 (83)
Diabetes	25 (50)	4 (31)	0 (0)	3 (17)
Obesity	11 (22)	4 (31)	18 (100)	6 (33)
Smoking	6 (12)	1 (8)	6 (33)	3 (17)
History of, n (%)				
CAD	22 (44)	5 (39)	0 (0)	3 (17)
Stroke/TIA	6 (12)	1 (8)	0 (0)	1 (6)
PAD	4 (8)	1 (8)	0 (0)	0 (0)
CKD	23 (46)	3 (25)	1 (6)	1 (6)
AF	29 (58)	6 (50)	1 (6)	1 (6)
Medications, n (%)				
ACEi	8 (16)	3 (25)	0 (0)	1 (6)
ARB	11 (22)	4 (33)	1 (6)	12 (67)
ARNI	5 (10)	1 (8)	0 (0)	0 (0)
CCB	8 (16)	6 (50)	2 (11)	10 (56)
Statin	29 (58)	9 (69)	3 (17)	15 (83)
B-blocker	34 (68)	8 (67)	4 (22)	7 (39)
MRA	26 (52)	5 (39)	1 (6)	1 (6)
Loop diuretic	37 (74)	8 (67)	1 (6)	0 (0)
Thiazide diuretic	0 (0)	2 (16)	0 (0)	6 (33)
Anticoagulant	31 (62)	5 (39)	0 (0)	0 (0)
Antiplatelet	15 (30)	5 (39)	0 (0)	1 (6)
SBP, mmHg*	130±25	133±16	139±14	149±22
DBP, mmHg*	70±14	67±11	84±11	87±13
BMI, kg/m^2*	27.2±4.8	28.6±4.0	35.1±3.5	29.9±4.4

6MWD, m*	263±151	362±165	531±121	492±103
Hb, g/dl*	12.5±2.2	13.3±2.3	14.3±1.9	14.3±1.2
Glucose, mg/dl**	138 (106, 168)	98 (90, 157)	94 (90, 106)	101 (94, 107)
eGFR, ml/min/1.73m^2*	55.1±18.5	64.5 ±17.4	82.2±22.9	80.0±13.5
TCHOL, mg/dl*	154±36	184 ± 52	219±31	180±53
HDL, mg/dl*	43±13	48±13	55±10	55±13
TRG, mg/dl*	125±65	129±63	113±44	109±42
LDL, mg/dl*	85±29	111±43	142±33	104±40†
Echo parameters*				
LVEF, %	38±19	45±15	60±2	60±3
LVIDD, mm	57±12	52±10	48±5	43±6
RWT	0.42±0.16	0.47±0.13	0.42±0.09	0.54±0.12
LVH, n (%)	31 (62)	6 (46)	4 (22)	7 (39)
E, cm/s	102±42	99±27	76±19	69±14
E/E'**	15.0 (12.8, 16.0)	12.0 (8.8, 13.5)	7.5 (6.8, 9.0)	7.0 (6.9, 9.0)
TRVmax, m/s	3.0±0.8	2.6±0.7	2.1±0.6	1.9±0.5
SerumNT-proBNP**	4706 (1237, 8438)	1232 (566, 3509)	64 (60, 88)	98 (63, 122)
Saliva NT-proBNP**	15.1 (10.4, 30.6)	8.6 (7.1, 35.2)	8.8 (6.3, 22.7)	11.1 (6.0, 17.6)
Serum Cortisol**	21.26 (2.2, 61.4)	10.14 (1.6, 23.3)	9.82 (5.7, 17.2)	10.89 (5.7, 22.3)
Saliva Cortisol**	5.62 (0.05, 15.63)	6 (0.37, 14.82)	4.07 (0.41, 11.08)	3.2 (0.98, 12.94)
*mean ± standard deviation **median (IQR)				

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

Table 11. Variables for which statistically significant differences were found between Group III and Group IV			
	Obese n=18 Group III	Hypertensive n=18 Group IV	**P value**
Age, years*	50±11	65±10	**0.001**
Weight, kg*	102±17	88±18	**0.012**
BMI,*	35.1±3.5	29.9±4.4	**0.0001**
Urea, mg/dL*	27.5±7	36.5±10	**0.002**
Cholesterol, mg/dL*	219±31	180±53	**0.027**
LDL, mg/dL*	142±33	104±40	**0.008**
LVIDD, mm*	48±5	43±6	**0.025**
RWT*	0.42±0.09	0.54±0.12	**0.0001**
E/A ratio*	1.05±0.32	0.79±0.2	**0.006**
Serum NT-proBNP, pg/mL**	64 (60, 114)	96 (60,186)	**0.025**

BMI= body mass index, LDL= low-density lipoprotein cholesterol, LVIDD= left ventricle internal diastolic diameter, RWT= regional wall thickness. *mean±SD, **median (IQR)

Table 12. Comparison among Obese, Hypertensive and CHF patients (Groups II, III, IV) at timepoint 0				
	Chronic HF, n=12 Group II	**Obese, n=18 Group III**	**Hypertensive, n=18 Group IV**	**P value, Kruskal Wallis test**
Age, years*	72±9	50±11	65±10	**<0.001**
Male gender, n (%)	7 (54)	8 (44)	9 (50)	0.870
CV risk factors, n (%)				
Hypertension	10 (77)	5 (28)	18 (100)	**<0.001**
Dyslipidemia	11 (85)	7 (3)	15 (83)	**0.005**
Diabetes	4 (31)	0 (0)	3 (17)	0.051
Obesity	4 (31)	18 (100)	6 (33)	**<0.001**
Smoking	1 (8)	6 (33)	3 (17)	0.265
History, n (%)				
CAD	5 (39)	0 (0)	3 (17)	**0.017**
Stroke/TIA	1 (8)	0 (0)	1 (6)	0.522
PAD	1 (8)	0 (0)	0 (0)	0.243
CKD	3 (23)	1 (6)	1 (6)	**0.012**
AF	6 (46)	1 (6)	1 (6)	**0.003**
Medications, n (%)				
ACEi	3 (23)	0 (0)	1 (6)	0.06
ARBs	4 (31)	1 (6)	12 (67)	**0.001**
CCB	6 (46)	2 (11)	10 (56)	**0.016**
Statins	9 (69)	3 (17)	15 (83)	**<0.001**
B-blockers	8 (62)	4 (22)	7 (39)	0.086
MRA	5 (39)	1 (6)	1 (6)	**0.015**

Loop diuretics	8 (62)	1 (6)	0 (0)	**<0.001**
Thiazide diuretics	2 (15)	0 (0)	6 (33)	**0.026**
SBP, mmHg*	133±16	139±14	149±22	**0.045**
DBP, mmHg*	67±11	84±11	87±13	**<0.001**
BMI, kg/m^2*	28.6±4.0	35.1±3.5	29.9±4.4	**<0.001**
6MWD, m*	362±165	531±121	492±103	**0.002**
Hb, g/dl*	13.3±2.3	14.3±1.9	14.3±1.2	0.220
Glucose, mg/dl**	98 (90, 157)	94 (90, 106)	101 (94, 107)	0.421
eGFR, ml/min/1.73m^2*	64.5±17.4	82.2±22.9	80.0±13.5	**0.026**
TCHOL, mg/dl*	184±52	219±31	180±53	**0.032**
HDL, mg/dl*	48±13	55±10	55±13	0.226
TRG, mg/dl*	129±63	113±44	109±42	0.505
LDL, mg/dl*	111±43	142±33	104±40	**0.032**
Echo parameters*				
LVEF, %	45±15	60±2	60±3	**<0.001**
LVIDD, mm	52±10	48±5	43±6	**0.004**
RWT	0.47±0.13	0.42±0.09	0.54±0.12	**0.011**
LVH, n (%)	6 (46)	4 (22)	7 (39)	0.345
E, cm/s	99±27	76±19	69±14	**0.001**
E/E'**	12.0 (8.8, 13.5)	7.5 (6.8, 9.0)	7.0 (6.9, 9.0)	**<0.001**
TRVmax, m/s	2.6±0.7	2.1±0.6	1.9±0.5	**0.009**
Serum NT-proBNP**	1232 (566, 3509)	64 (31, 93)	106 (63, 120)	**<0.001**
Saliva NT-proBNP**	8.6 (7.1, 35.2)	8.8 (6.3, 22.7)	11.1 (6.0, 17.6)	0.969

*mean±Standard Deviation, **median (IQR)

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB,

calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

Table 13. Comparison among AHF and CHF patients (Groups I, II)			
	Chronic HF, n=12 **Group II**	**Acute HF, n=49** **Group I**	**P value, Mann Whitney U test**
Age, years*	72±9	72±12	0.981
Male gender, n (%)	7 (54)	35 (70)	0.329
CV risk factors, n (%)			
Hypertension	10 (77)	43 (86)	0.417
Dyslipidemia	11 (85)	38 (76)	0.714
Diabetes	4 (31)	25 (50)	0.349
Obesity	4 (31)	11 (22)	0.489
Smoking	1 (8)	6 (12)	0.882
CVD History, n (%)			
CAD	5 (39)	22 (44)	0.764
Stroke/TIA	1 (8)	6 (12)	0.660
PAD	1 (8)	4 (8)	0.971
CKD	3 (23)	23 (46)	0.207
AF	6 (46)	29 (58)	0.537
Medications, n (%)			
ACEi	3 (23)	8 (16)	0.549
ARBs	4 (31)	11 (22)	0.508
ARNI	1 (8)	5 (10)	0.801
CCB	6 (46)	8 (16)	**0.020**
Statins	9 (69)	29 (58)	0.538
B-blockers	8 (62)	34 (68)	0.660
MRA	5 (39)	26 (52)	0.536
Loop diuretics	8 (62)	37 (74)	0.376

Anticoagulation	5 (39)	31 (62)	0.208
Antiplatelets	5 (39)	15 (30)	0.559
SBP, mmHg*	133±16	130±25	0.628
DBP, mmHg*	67±11	70±14	0.446
HR, bpm*	73±20	79±16	0.265
BMI, kg/m^2*	28.6±4.0	27.2±4.8	0.331
6MWD, m*	362±165	263±151	**0.045**
NYHA class (n%)			
I	1 (8)	0 (0)	
II	3 (23)	1 (2)	**0.003**
III	8 (61)	30 (60)	
IV	1 (8)	19 (38)	
QRS, msec**	118 (90, 159)	140 (99, 158)	0.535
WBC*	7238±2059	8499±2832	0.139
Hb, g/dl*	13.3±2.3	12.5±2.2	0.261
Glucose, mg/dl*	98 (90, 157)	138 (106, 168)	**0.036**
Urea, mg/dl*	51 (39, 80)	73 (54, 101)	**0.045**
eGFR, ml/min/1.73m^2*	64.5±17.4	55.1±18.5	0.107
K+*	4.05±0.41	4.24±0.52	0.216
Na+**	139 (138, 141)	138 (135, 140)	0.189
TCHOL, mg/dl*	184±52	154±36	**0.016**
HDL, mg/dl*	48±13	43±13	0.281
TRG, mg/dl*	129±63	125±65	0.844
LDL, mg/dl*	111±43	85±29	**0.013**
Echo parameters*			
LVEF, %	45±15	38±19	0.206
LVIDD, mm	52±10	57±12	0.176

RWT	0.47±0.13	0.42±0.16	0.366
LVH, n (%)	6 (46)	31 (62)	0.353
E, cm/s	99±27	102±42	0.775
E/E'**	12.0 (8.8, 13.5)	15.0 (12.8, 16.0)	**0.018**
TRVmax, m/s	2.6±0.7	3.0±0.8	0.131
IVCexp, mm	13 (10, 19)	23 (20, 25)	**0.001**
Serum NT-proBNP**	1232 (566, 3509)	4706 (1237, 8438)	**0.007**
Saliva NT-proBNP**	8.6 (7.1, 35.2)	15.1 (10.4, 30.6)	0.122
*mean ± standard deviation**median (IQR)			

Abbreviations: 6MWD, six minute walk distance; ACEi, angiotensin converting enzyme inhibitors; AF, atrial fibrillation; ARBs, angiotensin II receptor blockers; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; CAD, coronary artery disease; CCB, calcium channel blockers; CKD, chronic kidney disease; CV, cardiovascular; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; MRA, mineralocorticoid receptor antagonist; NYHA, New York Heart Association; PAD, peripheral artery disease; RWT, regional wall thickness; SBP, systolic blood pressure; TIA, transient ischemic attack.

Table 14. Comparison between serum NT-proBNP median (IQR) values among study groups and at all timepoints					
Variable 1	**Median (IQR)**	**Variable 2**	**Median (IQR)**	**Test**	**P value**
ALL Serum NT-proBNP at T0	844 (4810)	ALL Serum NT-proBNP at T4	527 (2401)	Wilcoxon, Signed Rank Test	0.158
AHF Serum NT-proBNP at T0	4493 (7136)	AHF Serum NT-proBNP at T1	2681.5 (5671)	Wilcoxon, Signed Rank Test	**0.002**
AHF Serum NT-proBNP at T0	4493 (7136)	AHF Serum NT-proBNP at T4	2873.5 (5442)	Wilcoxon, Signed Rank Test	0.061
AHF Serum NT-proBNP at T1	2681.5 (5671)	AHF Serum NT-proBNP at T4	2873.5 (5442)	Wilcoxon, Signed Rank Test	0.994
AHF Serum NT-proBNP at T0	4493 (7136)	CHF Serum NT-proBNP at T0	1221.5 (1818)	Mann Whitney U Test	**0.007**
AHF Serum NT-proBNP at T0	4493 (7136)	OB Serum NT-proBNP at T0	64 (33)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T0	4493 (7136)	HT Serum NT-proBNP at T0	110 (118)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T0	4493 (7136)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
AHF+CHF Serum NT-proBNP at T0	3826 (6213)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T0	1221.5 (1818)	CHF Serum NT-proBNP at T4	1115(1394)	Wilcoxon, Signed Rank Test	1.000
CHF Serum NT-proBNP at T0	1221.5 (1818)	OB Serum NT-proBNP at T0	64 (33)	Mann Whitney U Test	**0.001**

CHF Serum NT-proBNP at T0	1221.5 (1818)	HT Serum NT-proBNP at T0	110 (118)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T0	1221.5 (1818)	OB+HT Serum NT-proBNP at T0	75.5 (64)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	CHF Serum NT-proBNP at T4	1115(1394)	Mann Whitney U Test	**0.047**
AHF Serum NT-proBNP at T4	2873.5 (5442)	OB Serum NT-proBNP at T4	60.5 (63)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	HT Serum NT-proBNP at T4	112.5 (125)	Mann Whitney U Test	**0.001**
AHF Serum NT-proBNP at T4	2873.5 (5442)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
AHF+CHF Serum NT-proBNP at T4	2142.5 (4994)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	OB Serum NT-proBNP at T4	60.5 (63)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	HT Serum NT-proBNP at T4	112.5 (125)	Mann Whitney U Test	**0.001**
CHF Serum NT-proBNP at T4	1115(1394)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Mann Whitney U Test	**0.001**
OB Serum NT-proBNP at T0	64 (33)	OB Serum NT-proBNP at T4	60.5 (63)	Wilcoxon, Signed Rank Test	0.625
HT Serum NT-proBNP at T0	110 (118)	HT Serum NT-proBNP at T4	112.5 (125)	Wilcoxon, Signed Rank Test	0.691
OB+HT Serum NT-proBNP at T0	75.5 (64)	OB+HT Serum NT-proBNP at T4	88.5 (91)	Wilcoxon, Signed Rank Test	0.919

AHF+CHF Serum NT-proBNP at T0	3826 (6213)	AHF+CHF Serum NT-proBNP at T4	2142.5 (4994)	Wilcoxon, Signed Rank Test	0.086

AHF = acute HF; ALL = Total study population; CHF= chronic HF; HT=hypertensive; OB=obese; T0=day of admission or day of first exam; T1=day of discharge for AHF patients; T4 = four week follow-up.

Table 15. Comparison between salivary NT-proBNP median (IQR) values among study groups and at all timepoints.					
Variable 1	**Median (IQR)**	**Variable 2**	**Median (IQR)**	**Test**	**P value**
ALL Saliva NT-proBNP at T0	12.7 (16.3)	ALL Saliva NT-proBNP at T4	10.6 (0)	Wilcoxon, Signed Rank Test	0.354
AHF Saliva NT-proBNP at T0	15.1 (18.3)	AHF Saliva NT-proBNP at T1	10.7 (0)	Wilcoxon, Signed Rank Test	0.13
AHF Saliva NT-proBNP at T0	15.1 (18.3)	AHF Saliva NT-proBNP at T4	14.3 (24)	Wilcoxon, Signed Rank Test	0.457
AHF Saliva NT-proBNP at T1	10.7 (0)	AHF Saliva NT-proBNP at T4	14.3 (24)	Wilcoxon, Signed Rank Test	0.727
AHF Saliva NT-proBNP at T0	15.1 (18.3)	HT Saliva NT-proBNP at T0	11.1 (11.6)	Mann Whitney U Test	**0.032**
AHF Saliva NT-proBNP at T0	15.1 (18.3)	CHF Saliva NT-proBNP at T0	8.2 (0)	Mann Whitney U Test	0.162
AHF Saliva NT-proBNP at T0	15.1 (18.3)	OB Saliva NT-proBNP at T0	8.8 (0)	Mann Whitney U Test	0.062
AHF Saliva NT-proBNP at T0	15.1 (18.3)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	**0.012**
AHF+CHF Saliva NT-proBNP at T0	14.3 (22.3)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	**0.028**
AHF Saliva NT-proBNP at T4	14.3 (24)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Mann Whitney U Test	**0.037**
AHF Saliva NT-proBNP at T4	14.3 (24)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	**0.031**
AHF+CHF Saliva NT-proBNP at T4	13.8 (24.6)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	**0.033**

CHF Saliva NT-proBNP at T0	8.2 (0)	CHF Saliva NT-proBNP at T4	8.2 (0)	Wilcoxon, Signed Rank Test	0.674
OB Saliva NT-proBNP at T0	8.8 (0)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Wilcoxon, Signed Rank Test	0.446
HT Saliva NT-proBNP at T0	11.1 (11.6)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Wilcoxon, Signed Rank Test	0.619
OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Wilcoxon, Signed Rank Test	0.451
AHF+CHF Saliva NT-proBNP at T0	14.3 (22.3)	AHF+CHF Saliva NT-proBNP at T4	13.8 (24.6)	Wilcoxon, Signed Rank Test	0.527
CHF Saliva NT-proBNP at T0	8.2 (0)	OB Saliva NT-proBNP at T0	8.8 (0)	Mann Whitney U Test	0.851
CHF Saliva NT-proBNP at T0	8.2 (0)	HT Saliva NT-proBNP at T0	11.1 (11.6)	Mann Whitney U Test	0.819
CHF Saliva NT-proBNP at T0	8.2 (0)	OB+HT Saliva NT-proBNP at T0	10.1 (11.1)	Mann Whitney U Test	0.812
AHF Saliva NT-proBNP at T4	14.3 (24)	CHF Saliva NT-proBNP at T4	8.2 (0)	Mann Whitney U Test	0.928
AHF Saliva NT-proBNP at T4	14.3 (24)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Mann Whitney U Test	0.168
CHF Saliva NT-proBNP at T4	8.2 (0)	OB Saliva NT-proBNP at T4	6.5 (9.1)	Mann Whitney U Test	0.495
CHF Saliva NT-proBNP at T4	8.2 (0)	HT Saliva NT-proBNP at T4	7.4 (6.8)	Mann Whitney U Test	0.397

CHF Saliva NT-proBNP at T4	8.2 (0)	OB+HT Saliva NT-proBNP at T4	7.6 (8.7)	Mann Whitney U Test	0.393

AHF = acute HF; ALL = Total study population; CHF= chronic HF; HT=hypertensive; OB=obese; T0=day of admission or day of first exam; T1=day of discharge for AHF patients; T4 = four week follow-up.

Table 16. Linear correlation between serum and salivary NT-proBNP mean values				
Variable 1	**Variable 2**	**Correlation Coefficient**	**R value**	**P value**
ALL Serum NT-proBNP at T0	ALL Saliva NT-proBNP at T0	Spearman	**0.336**	**0.001**
ALL Serum NT-proBNP at T4	ALL Saliva NT-proBNP at T4	Spearman	0.170	0.118
AHF Serum NT-proBNP at T0	AHF Saliva NT-proBNP at T0	Spearman	**0.341**	**0.016**
AHF Serum NT-proBNP at T1	AHF Saliva NT-proBNP at T1	Pearson	**0.399**	**0.005**
AHF Serum NT-proBNP at T4	AHF Saliva NT-proBNP at T4	Pearson	-.093	0.557
AHF+CHF Serum NT-proBNP at T0	AHF+CHF Saliva NT-proBNP at T0	Pearson	**0.306**	**0.016**
AHF+CHF Serum NT-proBNP at T4	AHF+CHF Saliva NT-proBNP at T4	Pearson	-.052	0.721
CHF Serum NT-proBNP at T0	CHF Saliva NT-proBNP at T0	Spearman	0.294	0.354
CHF Serum NT-proBNP at T4	CHF Saliva NT-proBNP at T4	Spearman	-.381	0.352
OB Serum NT-proBNP at T0	OB Saliva NT-proBNP at T0	Spearman	0.213	0.397
OB Serum NT-proBNP at T4	OB Saliva NT-proBNP at T4	Spearman	0.260	0.917
HT Serum NT-proBNP at T0	HT Saliva NT-proBNP at T0	Spearman	-.119	0.638
HT Serum NT-proBNP at T4	HT Saliva NT-proBNP at T4	Spearman	-.091	0.720
OB+HT Serum NT-proBNP at T0	OB+HT Saliva NT-proBNP at T0	Spearman	0.056	0.746
OB+HT Serum NT-proBNP at T4	OB+HT Saliva NT-proBNP at T4	Spearman	-.013	0.938

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam; T1 = day of discharge for AHF patients; T4 = four week follow-up.*

Table 17. Correlations of serum and salivary NT-proBNP in AHF sub-populations at timepoint 0.			
Variable	**Characteristic**	**Correlation Coefficient**	**P Value**
Age, years	≤75	Spearman	0.102
	>75	Spearman	**0.047**
Sex	Male	Spearman	0.178
	Female	Spearman	0.176
eGFR mL/min/1.73m^2	30-44	Spearman	**0.07**
	45-59	Spearman	0.468
	>60	Spearman	0.875
BMI, kg/m^2	≤25	Spearman	0.088
	25-30	Spearman	0.221
	>30	Spearman	0.154
LVEF, %	≤40	Spearman	0.062
	>40	Spearman	0.111
AF	YES	Spearman	**0.002**
	NO	Spearman	0.376
NYHA	III	Spearman	0.295
	IV	Spearman	0.815
RAAS	NO	Spearman	**0.002**
B -BLOCKER	YES	Spearman	**0.005**
LVH	NO	Spearman	**0.046**
WEIGHT, kg	≤85	Spearman	**0.030**
6MWD, m	≤315	Spearman	**0.003**
WBC	≤8.4 X10^3	Spearman	**0.034**
Hb, g/dL	≤13.1	Spearman	**0.029**
Creatinine, mg/dL	>1.105	Spearman	**0.017**
Sodium, mEq/L	≤135	Spearman	**0.002**
Glucose, mg/dL	≥124	Spearman	**0.007**
Ferritin, ng/mL	≥23	Spearman	**0.030**
TSH, µU/mL	≤5.33	Spearman	**0.018**
LVIDD, mm	≥51.5	Spearman	**0.019**

E velocity, m/sec	>1.16	Spearman	**0.042**
TR Vmax, m/sec	≥2.8	Spearman	**0.007**
IVC inspiration, mm	≤21	Spearman	**0.032**

Abbreviations: 6MWD, six minute walk distance; AF, atrial fibrillation; BMI, body mass index; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IVC, inferior vena cava; LVEF, left ventricle ejection fraction; LVH, left ventricular hypertrophy; LVIDD, left ventricular internal end diastolic diameter; NYHA, New York Heart Association; RAAS: renin-angiotensin-aldosterone system; TRVmax: tricuspid valve regurgitation velocity; TSH, thyroid-stimulating hormone; WBC,white lood cell count.

Table 18. Non linear correlation between serum and salivary NT-proBNP values at T0						
Group	**Model with constant term**	**R square**	**P value**	**Model without constant term**	**R square**	**P value**
I (AHF)	Cubic	0.138	0.08	Power	0.934	**0.001**
II (CHF)	Cubic	0.603	**0.051**	Power	0.883	**0.001**
III (obese)	Cubic	0.842	**0.001**	Cubic	0.876	**0.001**
IV (hypertensive)	Cubic	0.16	0.471	Power	0.867	**0.001**

*dependent variable=Serum NT-proBNP, independent variable= Salivary NT-proBNP

Table *19*. Multiple Linear Regression / Group- AHF/ Predictors: All Variables at timepoint 0.													
method	FORWARD All Variables Entered as explanatory												
in. var	T0_SE_NT-proBNP							T0_SA_NT-proBNP					
	model includes constant		VIF	model without constant				model includes constant			model without constant		
	Model	Values		Model	Values	VIF		Model	Values	VIF	Model	Values	VIF
	Constant	-5525,140						Constant	-39,990				
	NYHA	3356,043	1,074	NYHA	3285,411	24,612		Ur	0,365	1,816	Ur	,298	4,829
	CKD	3086,069	1,369	CHOL	-33,328	15,062		eGFR	0,651	1,816	eGFR	,448	8,264
	Diuretic	-2705,456	1,288	Weight	-55,135	16,702					Age	-,330	14,972
				LVIDD	62,124	21,901							
R		0,706			0,922				0,540[b]			0,849	
R-Square		0,499			0,849				0,291			0,721	
A R-Square		0,458			0,833				0,254.			,699	
P-Value		**0,000**			**0,000**				**0,001**				
D-W		2,053			1,415				1,429			1,321	

A

Abbreviations: AF, atrial fibrillation; CHOL, cholesterol; D-W, CKD, chronic kidney disease; Durbin-Watson statistic; eGFR, estimated glomerular filtration rate; LAVI, left atrial volume index; LVIDD, left ventricular internal end diastolic diameter; NYHA, New York Heart Association; QRS, complex duration in msec; RAAS: renin-angiotensin-aldosterone system; TRVmax: tricuspid valve regurgitation velocity; Ur, urea; VIF, variance inflation factor; WBC,white lood cell count.

Table 20. Variables assessed for univariate correlations with saliva and serum NT-proBNP	
Somatometric parameters	Laboratory Investigations
Weight Body Mass index (BMI)	White Blood Ceel Count (WBC) Hemoglobin (Hb) Urea Creatinine Estimated Glomerular Filtration Rate (eGFR) Potassium (K+) Sodium (Na+) Glucose (Glu) Ferrum (Fe) Ferritin Thyroid Stimulating Hormone (TSH) Total Bilirubin (Tbil) Total Cholesterol (TCHOL) LDL cholesterol (LDL-c)
Vital signs	
Systolic Blood Pressure (SBP) Diastolic Blood Pressure (DBP) Heart Rate (HR) Breathing Rate (BR) Saturation O2	
Other Clinical Characteristics	
Presence of atrial fibrillation QRS duration in ECG NYHA functional class (I-IV) 6-Min Walking Distance (6MWD) EQ5D-5L score	
Medications	Echocardiographic parameters
RAAS inhibitors / ARNI MRA Diuretics Beta-Blockers	Left Ventricular Ejection Fraction (LVEF) Left Ventricle Internal End Diastolic Diameter (LVEDD) Left Ventricular mass (LV mass) LV hypertrophy (LVH) E velocity, E/A ratio, E/e' ratio TR Vmax IVC exp, IVC insp

Table 21. Significant (p<0.05) Univariate correlations of serum NT-proBNP with clinical, laboratory and echocardiographic parameters				
CHF patients at T0, n=12	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	WBC	Spearman	-0.601	**0.039**
	Creatinine	Spearman	0.592	**0.043**
	eGFR	Spearman	-0.664	**0.018**
	EQ5D-5L	Spearman	-0.664	**0.044**
CHF patients at T4, n=8	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	$SatO_2$	Spearman	0.727	**0.041**
	WBC	Spearman	0.714	**0.047**
	Ferritin	Spearman	0.775	**0.041**
	eGFR	Spearman	-0.976	**0.0001**
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.298	**0.037**
	Urea	Pearson	0.408	**0.004**
	$SatO_2$	Spearman	-0.339	**0.017**
	Creatinine	Pearson	0.385	**0.006**
	IVC exp	Spearman	0.378	**0.007**
	IVC ins	Pearson	0.363	**0.010**
	EQ5D-5L	Pearson	-0.327	**0.022**
	6MWD	Pearson	-0.399	**0.005**
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.294	**0.043**
	EQ5D-5L	Pearson	-0.287	**0.048**
	RAASi/ARNI use	Mann Whitney		**0.028**
	DBP	Pearson	-0.434	**0.002**

	TCHOL	Spearman	-0.69	**0.001**
	LDL-chol	Spearman	-0.618	**0.001**
	Hb	Pearson	-0.301	**0.038**
	Urea	Pearson	0.319	**0.027**
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.474	**0.002**
	LVH yes	Mann Whitney		**0.049**
	RAAS use	Mann Whitney		**0.018**
	Diuretic use	Mann Whitney		**0.036**
	BR	Spearman	0.401	**0.009**
	eGFR	Pearson	-0.428	**0.005**
	TCHOL	Spearman	-0.591	**0.0001**
	LDL-chol	Spearman	-0.522	**0.001**
	LA diam	Spearman	0.503	**0.039**
	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	Urea	Pearson	0.362	**0.019**
	Creatinine	Pearson	0.325	**0.035**
	TBIL	Pearson	0.352	**0.024**
AHF and CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	6MWD	Pearson	-0.411	**0.001**
	SBP	Pearson	-0.258	**0.045**
	IVC exp	Pearson	0.425	**0.001**
	Urea	Pearson	0.487	**0.0001**
	Creatinine	Pearson	0487	**0.0001**
	TCHOL	Pearson	-0.4	**0.001**
	IVC insp	Pearson	0.446	**0.0001**
	$SatO_2$	Spearman	-0.358	**0.005**
	eGFR	Pearson	-0.396	**0.002**
	TBIL	Pearson	0.302	**0.019**
	LDL-chol	Pearson	-0.361	**0.004**
	E/E' ratio	Pearson	0.263	**0.048**

	TR Vmax	Pearson	0.265	**0.039**
	EQ5D-5L	Pearson	-0.395	**0.002**
AHF and CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.43	**0.002**
	Urea	Pearson	0.471	**0.001**
	eGFR	Pearson	-0.524	**0.0001**
	TCHOL	Pearson	-0.542	**0.0001**
	LDL-chol	Pearson	-0454	**0.002**
	LA diam	Spearman	0.503	**0.039**
	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	TR Vmax	Spearman	0.446	**0.037**
	EQ5D-5L	Pearson	-0.408	**0.003**
	QRS	Pearson	0.280	**0.049**
	BR	Pearson	0.384	**0.006**
	Creatinine	Pearson	0.455	**0.001**
	LVH yes	Mann Whitney		**0.049**
	RAAS use	Mann Whitney		**0.041**
	Diuretic use	Mann Whitney		**0.006**

Abbreviations: 6MWD, six minute walk distance; ARNI, angiotensin receptor-neprilysin inhibitor; BR, breathing rate; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; Hb, hemoglobin; IVC exp, inferior vena cava diameter at expiration; IVC insp, inferior vena cava diameter at inspiration; LAdiam, left atrium diameter; LDL-chol, low-density lipoprotein; LVH, left ventricular hypertrophy; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; SBP, systolic blood pressure; TBIL, total bilirubin; TCHOL, total cholesterol; TRVmax, tricuspid regurgitation maximum velocity; WBC, white blood cell count.

Table 21 extended. All Univariate correlations of serum NT-proBNP with clinical, laboratory and echocardiographic parameters				
CHF patients at T0, n=12	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	WBC	Spearman	-0.601	**0.039**
	Creatinine	Spearman	0.592	**0.043**
	eGFR	Spearman	-0.664	**0.018**
	EQ5D-5L	Spearman	-0.664	**0.044**
	SBP	Spearman	0.321	0.309
	DBP	Spearman	-0.105	0.745
	QRS	Spearman	0.404	0.192
	HR	Spearman	-0.158	0.625
	BR	Spearman	0.224	0.485
	$SatO_2$	Spearman	-0.133	0.681
	Weight	Spearman	0.084	0.795
	BMI	Spearman	-0.32	0.923
	6MWD	Spearman	-0.238	0.457
	Hb	Spearman	-0.091	0.779
	Fe	Spearman	-0.246	0.446
	Ferritin	Spearman	0.382	0.247
	TSH	Spearman	0.091	0.790
	Urea	Spearman	0.516	0.086
	Sodium	Spearman	-0.089	0.794
	Potassium	Spearman	0.001	1.000
	TBIL	Spearman	0.309	0.356
	TCHOL	Spearman	-0.448	0.145
	LDL-chol	Spearman	-0.441	0.152
	Glu	Spearman	-0.095	0.770
	LVEF	Spearman	-0.212	0.508
	LVEDD	Spearman	-0.028	0.931
	LV mass	Spearman	0.250	0.516

	LA diam	Spearman	0.381	0.222
	LAVI	Spearman	0.336	0.286
	E velocity	Spearman	0.406	0.191
	E/A ratio	Spearman	0.131	0.718
	E/E' ratio	Spearman	-0.102	0.753
	TR Vmax	Spearman	0.105	0.745
	IVC exp	Spearman	-0.063	0.845
	IVC insp	Spearman	-0.113	0.726
	AF yes	Mann Whitney		1.000
	LVH yes	Mann Whitney		0.202
	RAASi/ARNI use	Mann Whitney		0.530
	b-blocker use	Mann Whitney		0.432
	MRA use	Mann Whitney		0.214
	Diuretic use	Mann Whitney		0.209
CHF patients at T4, n=8	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Spearman	-0.095	0.823
	DBP	Spearman	-0.476	0.233
	QRS	Spearman	0.476	0.233
	HR	Spearman	0.619	0.102
	BR	Spearman	-0.109	797
	Weight	Spearman	-0.133	0.754
	BMI	Spearman	0.190	0.651
	6MWD	Spearman	-0.611	0.108
	Hb	Spearman	-0.190	0.651
	Fe	Spearman	0.600	0.208
	TSH	Spearman	0.310	0.456
	Urea	Spearman	0.683	0.062
	Creatinine	Spearman	0.476	0.233
	Sodium	Spearman	-0.135	0.750
	Potassium	Spearman	0.238	0.570
	TBIL	Spearman	0.232	0.658
	TCHOL	Spearman	0.400	0.505
	LDL-chol	Spearman	0.300	0.624

	Glu	Spearman	0.241	0.565
	EQ5D-5L	Spearman	-0.699	0.054
	RAAS use	Mann Whitney		0.571
	MRA use	Mann Whitney		0.672
	$SatO_2$	Spearman	0.727	**0.041**
	WBC	Spearman	0.714	**0.047**
	Ferritin	Spearman	0.775	**0.041**
	eGFR	Spearman	-0.976	**0.0001**
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.298	**0.037**
	DBP	Pearson	-0.012	0.936
	QRS	Pearson	0.216	0.136
	HR	Pearson	-0.137	0.347
	BR	Pearson	0.040	0.787
	Weight	Pearson	-0.238	0.099
	BMI	Pearson	-0.222	0.126
	WBC	Pearson	0.100	0.494
	Hb	Pearson	-0.084	0.565
	Fe	Spearman	-0.129	0.381
	Ferritin	Spearman	-0.104	0.479
	TSH	Pearson	-0.063	0.667
	Urea	Pearson	0.408	**0.004**
	Sodium	Pearson	0.056	0.702
	Potassium	Pearson	0.024	0.869
	eGFR	Pearson	-0.294	0.041
	TBIL	Pearson	0.268	0.063
	TCHOL	Pearson	-0.327	0.022
	LDL-chol	Pearson	-0.261	0.070
	Glu	Pearson	-0.254	0.078
	LVEF	Pearson	-0.184	0.205
	LVEDD	Pearson	0.249	0.084
	LV mass	Pearson	-0.022	0.880
	LA diam	Pearson	0.198	0.221

	LAVI	Pearson	0.168	0.275
	E velocity	Pearson	-0.085	0.570
	E/A ratio	Spearman	0.343	0.101
	E/E' ratio	Spearman	0.190	0.211
	TR Vmax	Pearson	0.204	0.160
	$SatO_2$	Spearman	-0.339	**0.017**
	Creatinine	Pearson	0.385	**0.006**
	IVC exp	Spearman	0.378	**0.007**
	IVC ins	Pearson	0.363	**0.01**
	EQ5D-5L	Pearson	-0.327	**0.022**
	AF yes	Mann Whitney		0.801
	LVH yes	Mann Whitney		0.910
	RAASi/ARNI use	Mann Whitney		0.920
	b-blocker use	Mann Whitney		0.847
	MRA use	Mann Whitney		0.771
	Diuretic use	Mann Whitney		0.857
	6MWD	Pearson	-0.399	**0.005**
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.294	**0.043**
	QRS	Pearson	0.200	0.174
	HR	Pearson	-0.100	0.500
	BR	Pearson	-0.041	0.782
	$SatO_2$	Pearson	-0.129	0.384
	Weight	Pearson	-0.204	0.165
	BMI	Pearson	-0.162	0.270
	6MWD	Pearson	-0.167	0.261
	WBC	Pearson	0.142	0.336
	Creatinine	Pearson	0.209	0.154
	Sodium	Pearson	-0.130	0.379
	Potassium	Pearson	0.093	0.529
	TBIL	Pearson	0.199	0.201
	Glu	Pearson	-0.109	0.469
	EQ5D-5L	Pearson	-0.287	**0.048**

	AF yes	Mann Whitney		0.444
	RAASi/ARNI use	Mann Whitney		**0.028**
	b-blocker use	Mann Whitney		0.233
	MRA use	Mann Whitney		0.541
	DBP	Pearson	-0.434	**0.002**
	TCHOL	Spearman	-0.69	**0.001**
	LDL-chol	Spearman	-0.618	**0.001**
	Hb	Pearson	-0.301	**0.038**
	Urea	Pearson	0.319	**0.027**
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.474	**0.002**
	DBP	Pearson	-0.242	0.123
	QRS	Pearson	0.268	0.086
	HR	Pearson	0.174	0.270
	$SatO_2$	Pearson	-0.140	0.370
	Weight	Pearson	-0.221	0.160
	BMI	Pearson	-0.145	0.361
	6MWD	Pearson	-0.225	0.180
	WBC	Pearson	0.155	0.333
	Hb	Pearson	-0.180	0.261
	Fe	Spearman	0.017	0.916
	Ferritin	Spearman	0.006	0.970
	TSH	Pearson	0.225	0.163
	Sodium	Pearson	-0.073	0.648
	Glu	Pearson	0.069	0.663
	LVEF	Pearson	-0.279	0.209
	LVEDD	Pearson	0.228	0.309
	LV mass	Pearson	0.201	0.382
	LAVI	Pearson	0.370	0.099
	E/A ratio	Spearman	0.122	0.690
	TR Vmax	Pearson	0.446	0.037
	IVC exp	Pearson	0.126	0.576
	EQ5D-5L	Pearson	-0.392	0.10

	AF yes	Mann Whitney		0.124
	LVH yes	Mann Whitney		**0.049**
	RAAS use	Mann Whitney		**0.018**
	b-blocker use	Mann Whitney		0.604
	MRA use	Mann Whitney		0.869
	Diuretic use	Mann Whitney		**0.036**
	BR	Spearman	0.401	**0.009**
	eGFR	Pearson	-0.428	**0.005**
	TCHOL	Spearman	-0.591	**0.0001**
	LDL-chol	Spearman	-0.522	**0.001**
	LA diam	Spearman	0.503	**0.039**
	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	Urea	Pearson	0.362	**0.019**
	Creatinine	Pearson	0.325	**0.035**
	TBIL	Pearson	0.352	**0.024**
AHF and CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	6MWD	Pearson	-0.411	**0.001**
	SBP	Pearson	-0.258	**0.045**
	DBP	Pearson	0.008	0.954
	QRS	Pearson	0.243	0.059
	HR	Pearson	-0.037	0.781
	BR	Pearson	0.215	0.096
	Weight	Pearson	-0.213	0.099
	BMI	Pearson	-0.212	0.101
	WBC	Pearson	0.088	0.499
	Hb	Pearson	-0.153	0.239
	Fe	Spearman	-0.193	0.143
	Ferritin	Spearman	-0.049	0.712
	TSH	Pearson	-0.081	0.540
	Sodium	Pearson	-0.054	0.680
	Potassium	Pearson	0.084	0.519
	Glu	Pearson	-0.148	0.256

	LVEF	Pearson	-0.235	0.068
	LVEDD	Pearson	0.251	0.051
	LV mass	Pearson	0.036	0.787
	LA diam	Pearson	0.215	0.126
	LAVI	Pearson	0.236	0.080
	E velocity	Pearson	0.008	0.949
	E/A ratio	Spearman	0.321	0.064
	IVC exp	Pearson	0.425	**0.001**
	AF yes	Mann Whitney		0.808
	LVH yes	Mann Whitney		0.506
	RAAS use	Mann Whitney		0.767
	b-blocker use	Mann Whitney		0.738
	MRA use	Mann Whitney		0.301
	Diuretic use	Mann Whitney		0.434
	Urea	Pearson	0.487	**0.0001**
	Creatinine	Pearson	0487	**0.0001**
	TCHOL	Pearson	-0.4	**0.001**
	IVC insp	Pearson	0.446	**0.0001**
	SBP	Pearson	-0.258	**0.045**
	$SatO_2$	Spearman	-0.358	**0.005**
	eGFR	Pearson	-0.396	**0.002**
	TBIL	Pearson	0.302	**0.019**
	LDL-chol	Pearson	-0.361	**0.004**
	E/E' ratio	Pearson	0.263	**0.048**
	TR Vmax	Pearson	0.265	**0.039**
	EQ5D-5L	Pearson	-0.395	**0.002**
AHF and CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	SBP	Pearson	-0.43	**0.002**
	Urea	Pearson	0.471	**0.001**
	eGFR	Pearson	-0.524	**0.0001**
	TCHOL	Pearson	-0.542	**0.0001**
	LDL-chol	Pearson	-0454	**0.002**
	LA diam	Spearman	0.503	**0.039**

	E velocity	Spearman	0.456	**0.033**
	E/E' ratio	Spearman	0.508	**0.022**
	TR Vmax	Spearman	0.446	**0.037**
	EQ5D-5L	Pearson	-0.408	**0.003**
	QRS	Pearson	0.280	**0.049**
	DBP	Pearson	-0.271	0.057
	HR	Pearson	0.223	0.120
	BR	Pearson	0.384	**0.006**
	$SatO_2$	Spearman	0.70	0.629
	Weight	Pearson	-0.157	0.276
	BMI	Pearson	-0.111	0.443
	6MWD	Pearson	-0.318	0.033
	WBC	Spearman	0.225	0.121
	Hb	Pearson	-0.181	0.214
	Fe	Pearson	0.065	0.672
	Ferritin	Spearman	0.123	0.412
	TSH	Pearson	0.247	0.090
	Creatinine	Pearson	0.455	**0.001**
	Sodium	Pearson	-0.164	0.256
	Potassium	Pearson	0.020	0.890
	Glu	Pearson	0.1220	0.398
	LVEF	Spearman	-0.279	0.209
	LVEDD	Spearman	0.228	0.309
	LV mass	Spearman	0.201	0.382
	LAVI	Spearman	0.370	0.099
	E/A wave	Spearman	0.122	0.690
	IVC exp	Spearman	0.126	0.576
	IVC insp	Spearman	0.280	0.208
	AF yes	Mann Whitney		0.091
	LVH yes	Mann Whitney		**0.049**
	RAAS use	Mann Whitney		**0.041**
	b-blocker use	Mann Whitney		0.971
	MRA use	Mann Whitney		0.933
	Diuretic use	Mann Whitney		**0.006**

Abbreviations: 6MWD, six minute walk distance; AF= atrial fibrillation; ARNI, angiotensin receptor-neprilysin inhibitor; BMI= body mass index; BR, breathing rate; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; Fe, ferrum; Glu, glucose; Hb, hemoglobin; HR, heart rate; IVC exp, inferior vena cava diameter at expiration; IVC insp, inferior vena cava diameter at inspiration; LAdiam, left atrium diameter; LAVI=left atrial volume index; LDL-chol, low-density lipoprotein; LVEDD=left ventricular end diastolic diameter; LVEF=left ventricle ejection fraction; LVH, left ventricular hypertrophy; MRA= mineralocorticoid receptor antagonist; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; SBP, systolic blood pressure; TBIL, total bilirubin; TCHOL, total cholesterol; TRVmax, tricuspid regurgitation maximum velocity; TSH, thyroid-stimulating hormone; WBC, white blood cell count.

Table 22.Significant (p<0.05) and near significant (p<0.1) Univariate correlations of salivary NT-proBNP with clinical, laboratory and echocardiographic parameters.				
CHF patients at T0, n=12	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Spearman	0.605	**0.037**
	Weight	Spearman	0.636	**0.026**
	LV mass	Spearman	0.7	**0.036**
	HR	Spearman	-0.62	**0.032**
	EQ5D-5L	Spearman	-0.523	0.081
CHF patients at T4, n=8	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Spearman	0.735	**0.038**
	RAAS use	Levene's		0.071
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Creatinine	Spearman	0.294	**0.040**
	TBIL	Spearman	0.298	**0.038**
	E/A ratio	Spearman	0.468	**0.021**
	Urea	Spearman	0.247	0.087
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	HR	Pearson	-0.323	**0.027**
	6MWD	Pearson	-0.255	0.088
	MRA use	Mann Whitney		**0.045**
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	BMI	Pearson	0.371	**0.016**
	IVC exp	Spearman	0.404	0.062

	Diuretic use	Mann Whitney		**0.048**
AHF+CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Spearman	0.248	0.054
	LV mass	Spearman	0.236	0.075
	BR	Spearman	0.230	0.075
	6MWD	Spearman	-0.276	**0.033**
	Urea	Spearman	0.233	0.071
	Creatinine	Spearman	0.345	**0.006**
	TBIL	Spearman	0.252	0.052
	E/A ratio	Spearman	0.378	**0.027**
	IVC exp	Spearman	0.273	**0.033**
	EQ5D-5L	Spearman	-0.250	0.052
AHF+CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Pearson	0.306	**0.031**
	$SatO_2$	Spearman	-0.309	**0.029**
	BMI	Pearson	0.392	**0.005**
	IVC exp	Spearman	0.404	0.062

Abbreviations: 6MWD, six minute walk distance; ARNI, angiotensin receptor-neprilysin inhibitor; BMI, body mass index; BR, breathing rate; HR, heart rate; IVC exp, inferior vena cava diameter at expiration; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; TBIL, total bilirubin.

Table 22 extended. All Univariate correlations of salivary NT-proBNP with clinical, laboratory and echocardiographic parameters.				
CHF patients at T0, n=12	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Spearman	0.605	**0.037**
	Weight	Spearman	0.636	**0.026**

	LV mass	Spearman	0.7	**0.036**
	HR	Spearman	-0.62	**0.032**
	SBP	Pearson	0.148	0.647
	DBP	Spearman	0.039	0.905
	BR	Spearman	0.374	0.231
	$SatO_2$	Spearman	0.051	0.875
	BMI	Spearman	0.480	0.114
	6MWD	Spearman	-0.427	0.167
	WBC	Spearman	-0.357	0.255
	Hb	Spearman	0.315	0.319
	Fe	Spearman	0.260	0.441
	Ferritin	Spearman	-0.073	0.832
	TSH	Spearman	0.200	0.555
	Urea	Spearman	-0.147	0.648
	Creatinine	Spearman	0.284	0.372
	Sodium	Spearman	-0.60	0.853
	Potassium	Spearman	0.028	0.931
	eGFR	Spearman	0.154	0.633
	TBIL	Spearman	0.032	0.925
	TCHOL	Spearman	0.035	0.914
	LDL-chol	Spearman	0.70	0.829
	Glu	Spearman	-0.165	0.609
	LVEF	Spearman	-0.544	0.167
	LVEDD	Spearman	0.434	0.158
	LA diam	Spearman	0.325	0.303
	LAVI	Spearman	0.322	0.308
	E velocity	Spearman	0.208	0.516
	E/A ratio	Spearman	0.038	0.918
	E/E’ ratio	Spearman	0.334	0.289
	TR Vmax	Spearman	-0.028	0.931
	IVC exp	Spearman	-0.063	0.845

	IVC insp	Spearman	0.265	0.405
	EQ5D-5L	Spearman	-0.523	0.081
	AF yes	Mann Whitney		0.282
	LVH yes	Mann Whitney		0.639
	RAASi/ARNI use	Mann Whitney		0.755
	B-Blocker use	Mann Whitney		0.876
	MRA use	Mann Whitney		0.461
	Diuretic use	Mann Whitney		0.600
CHF patients at T4, n=8	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	Weight	Spearman	0.735	**0.038**
	QRS	Spearman	0.381	0.352
	HR	Spearman	-0.286	0.493
	SBP	Spearman	0.357	0.3850
	DBP	Spearman	0.286	0.493
	BR	Spearman	0.327	0.429
	$SatO_2$	Spearman	-0.230	0.583
	BMI	Spearman	0.500	0.207
	6MWD	Spearman	0.347	0.399
	WBC	Spearman	-0.381	0.352
	Hb	Spearman	-0.238	0.570
	Fe	Spearman	-0.314	0.544
	Ferritin	Spearman	0.001	1.000
	TSH	Spearman	0.143	0.736
	Urea	Spearman	-0.072	0.866
	Creatinine	Spearman	0.286	0.493
	Sodium	Spearman	-0.381	0.352
	Potassium	Spearman	0.524	0.183
	eGFR	Spearman	0.310	0.456
	TBIL	Spearman	-0.638	0.173
	TCHOL	Spearman	0.300	0.624

	LDL-chol	Spearman	0.100	0.873
	Glu	Spearman	0.048	0.910
	EQ5D-5L	Spearman	0.24	0.955
	RAAS use	Levene's		0.071
	B-Blocker use	Levene's		0.200
	MRA use	Mann Whitney		0.571
AHF patients at T0, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Spearman	0.024	0.868
	Weight	Spearman	0.203	0.163
	LV mass	Spearman	0.151	0.301
	HR	Spearman	-0.023	0.878
	SBP	Pearson	-.0201	0.165
	DBP	Spearman	-0.044	0.762
	BR	Spearman	0.171	0.239
	$SatO_2$	Spearman	-0.158	0.278
	BMI	Spearman	0.055	0.707
	6MWD	Spearman	-0.227	0.121
	WBC	Spearman	0.031	0.832
	Hb	Spearman	0.226	0.118
	Fe	Spearman	0.077	0.601
	Ferritin	Spearman	0.091	0.535
	TSH	Spearman	0.103	0.480
	Urea	Spearman	0.247	0.087
	Creatinine	Spearman	0.294	**0.040**
	Sodium	Spearman	0.072	0.623
	Potassium	Spearman	-0.053	0.716
	eGFR	Spearman	-0.155	0.287
	TBIL	Spearman	0.298	**0.038**
	TCHOL	Spearman	-0.209	0.149
	LDL-chol	Spearman	-0.153	0.293

	Glu	Spearman	-0.135	0.355
	LVEF	Spearman	0.001	0.992
	LVEDD	Spearman	0.085	0.564
	LA diam	Spearman	0.018	0.910
	LAVI	Spearman	0.125	0.4190
	E velocity	Spearman	0.084	0.575
	E/A ratio	Spearman	0.468	**0.021**
	E/E' ratio	Spearman	0.050	0.742
	TR Vmax	Spearman	0.184	0.207
	IVC exp	Spearman	0.254	0.079
	IVC insp	Spearman	0.098	0.505
	EQ5D-5L	Spearman	-0.119	0.414
	AF yes	Mann Whitney		0.620
	LVH yes	Mann Whitney		0.295
	RAASi/ARNI use	Mann Whitney		0.704
	B-Blocker use	Mann Whitney		0.670
	MRA use	Mann Whitney		0.346
	Diuretic use	Mann Whitney		0.402
AHF patients at T1, n=49	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Pearson	0.021	0.891
	Weight	Pearson	0.009	0.953
	HR	Pearson	-0.323	**0.027**
	SBP	Spearman	-0.049	0.746
	DBP	Pearson	-0.162	0.277
	BR	Pearson	-0.155	0.300
	$SatO_2$	Spearman	-0.236	0.110
	BMI	Pearson	0.076	0.611
	6MWD	Pearson	-0.255	0.088
	WBC	Pearson	0.037	0.805
	Hb	Pearson	-0.111	0.457

	Urea	Pearson	0.129	0.388
	Creatinine	Pearson	0.129	0.388
	Sodium	Pearson	-0.044	0.770
	Potassium	Pearson	0.086	0.565
	eGFR	Pearson	-0.094	0.529
	TBIL	Pearson	0.072	0.650
	TCHOL	Pearson	-0.229	0.215
	LDL-chol	Pearson	-0.253	0.203
	Glu	Pearson	-0.020	0.899
	EQ5D-5L	Pearson	-0.133	0.374
	AF yes	Mann Whitney		0.426
	RAAS use	Mann Whitney		0.898
	B-Blocker	Mann Whitney		0.564
	MRA use	Mann Whitney		0.104
AHF patients at T4, n=42	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Pearson	-0.190	0.227
	Weight	Pearson	0.235	0.135
	LV mass	Spearman	-0.082	0.704
	HR	Pearson	-0.033	0.835
	SBP	Pearson	0.194	0.218
	DBP	Pearson	0.009	0.955
	BR	Pearson	-0.059	0.709
	$SatO_2$	Pearson	-0.248	0.113
	BMI	Pearson	0.371	**0.016**
	6MWD	Pearson	0.003	0.986
	WBC	Pearson	-0.065	0.687
	Hb	Pearson	0.055	0.733
	Fe	Spearman	0.038	0.816
	Ferritin	Spearman	-0.046	0.778
	TSH	Pearson	-0.059	0.717

	Urea	Pearson	0.023	0.884
	Creatinine	Pearson	-0.071	0.655
	Sodium	Pearson	-0.196	0.214
	Potassium	Pearson	0.114	0.474
	GFR	Pearson	0.023	0.866
	TBIL	Pearson	-0.011	0.946
	TCHOL	Spearman	0.040	0.808
	LDL-chol	Spearman	0.086	0.608
	Glu	Pearson	-0.098	0.536
	LVEF	Spearman	0.208	0.352
	LVEDD	Spearman	-0.032	0.889
	LA diam	Spearman	-0.029	0.912
	LAVI	Spearman	-0.159	0.490
	E velocity	Spearman	-0.042	0.852
	E/A ratio	Spearman	0.133	0.665
	E/E' ratio	Spearman	-0.146	0.540
	TR Vmax	Spearman	-0.025	0.911
	IVC exp	Spearman	0.404	0.062
	IVC insp	Spearman	0.245	0.272
	EQ5D-5L	Pearson	0.080	0.614
	AF yes	Mann Whitney		0.865
	LVH yes	Mann Whitney		0.449
	RAAS use	Mann Whitney		0.980
	B-Blocker use	Mann Whitney		0.352
	MRA use	Mann Whitney		0.335
	Diuretic use	Mann Whitney		**0.048**
AHF+CHF patients at T0, n=61	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Spearman	0.164	0.207
	Weight	Spearman	0.248	0.054
	LV mass	Spearman	0.236	0.075

	HR	Spearman	-0.105	0.420
	SBP	Spearman	-0.154	0.235
	DBP	Spearman	-0.033	0.801
	BR	Spearman	0.230	0.075
	$SatO_2$	Spearman	-0.145	0.264
	BMI	Spearman	0.095	0.467
	6MWD	Spearman	-0.276	**0.033**
	WBC	Spearman	-0.010	0.936
	Hb	Spearman	0.135	0.298
	Fe	Spearman	0.061	0.645
	Ferritin	Spearman	0.024	0.854
	TSH	Spearman	0.149	0.257
	Urea	Spearman	0.233	0.071
	Creatinine	Spearman	0.345	**0.006**
	Sodium	Spearman	0.004	0.974
	Potassium	Spearman	-0.034	0.794
	GFR	Spearman	-0.174	0.179
	TBIL	Spearman	0.252	0.052
	TCHOL	Spearman	-0.188	0.147
	LDL-chol	Spearman	-0.199	0.125
	Glu	Spearman	-0.91	0.484
	LVEF	Spearman	-0.130	0.317
	LVEDD	Spearman	0.166	0.201
	LA diam	Spearman	0.084	0.552
	LAVI	Spearman	0.193	0.153
	E velocity	Spearman	0.162	0.220
	E/A ratio	Spearman	0.378	**0.027**
	E/E’ ratio	Spearman	0.208	0.121
	TR Vmax	Spearman	0.188	0.147
	IVC exp	Spearman	0.273	**0.033**
	IVC insp	Spearman	0.197	0.128

	EQ5D-5L	Spearman	-0.250	0.052
	AF yes	Mann Whitney		0.367
	LVH yes	Mann Whitney		0.821
	RAAS use	Mann Whitney		0.834
	B-Blocker use	Mann Whitney		0.779
	MRA use	Mann Whitney		0.480
	Diuretic use	Mann Whitney		0.613
AHF+CHF patients at T4, n=50	**Variable**	**Correlation Coefficient**	**R value**	**P value**
	QRS	Pearson	-0.040	0.781
	Weight	Pearson	0.306	**0.031**
	LV mass	Spearman	-0.082	0.724
	HR	Pearson	-0.162	0.260
	SBP	Pearson	0.223	0.119
	DBP	Pearson	0.088	0.542
	BR	Pearson	0.003	0.983
	$SatO_2$	Spearman	-0.309	**0.029**
	BMI	Pearson	0.392	**0.005**
	6MWD	Pearson	0.088	0.563
	WBC	Spearman	-0.123	0.401
	Hb	Pearson	0.064	0.662
	Fe	Pearson	0.079	0.606
	Ferritin	Spearman	-0.031	0.836
	TSH	Pearson	-0.029	0.845
	Urea	Pearson	0.022	0.879
	Creatinine	Pearson	0.052	0.721
	Sodium	Pearson	-0.146	0.311
	Potassium	Pearson	0.081	0.577
	GFR	Pearson	0.011	0.938
	TBIL	Pearson	-0.032	0.829
	TCHOL	Pearson	0.242	0.113

	LDL-chol	Pearson	0.228	0.141
	Glu	Pearson	-0.117	0.419
	LVEF	Spearman	0.208	0.352
	LVEDD	Spearman	-0.032	0.889
	LA diam	Spearman	-0.029	0.912
	LAVI	Spearman	-0.159	0.490
	E velocity	Spearman	-0.042	0.852
	E/A ratio	Spearman	0.133	0.665
	E/E' ratio	Spearman	-0.146	0.540
	TR Vmax	Spearman	-0.025	0.911
	IVC exp	Spearman	0.404	0.062
	IVC insp	Spearman	0.245	0.272
	EQ5D-5L	Pearson	0.074	0.609
	AF yes	Mann Whitney		0.852
	LVH yes	Mann Whitney		0.449
	RAASi/ARNI use	Mann Whitney		0.807
	B-Blocker use	Mann Whitney		0.542
	MRA use	Mann Whitney		0.274
	Diuretic use	Mann Whitney		0.190

Abbreviations: 6MWD, six minute walk distance; AF= atrial fibrillation; ARNI, angiotensin receptor-neprilysin inhibitor; BMI= body mass index; BR, breathing rate; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; Fe, ferrum; Glu, glucose; Hb, hemoglobin; HR, heart rate; IVC exp, inferior vena cava diameter at expiration; IVC insp, inferior vena cava diameter at inspiration; LAdiam, left atrium diameter; LAVI=left atrial volume index; LDL-chol, low-density lipoprotein; LVEDD=left ventricular end diastolic diameter; LVEF=left ventricle ejection fraction; LVH, left ventricular hypertrophy; MRA= mineralocorticoid receptor antagonist; QRS, complex duration in electrocardiogram; RAASi, rennin-angiotensine-aldosterone system inhibitors; $SatO_2$, oxygen saturation; SBP, systolic blood pressure; TBIL, total bilirubin; TCHOL, total cholesterol; TRVmax, tricuspid regurgitation maximum velocity; TSH, thyroid-stimulating hormone; WBC, white blood cell count.

Table 23. Comparison between Serum Cortisol values among study groups at timepoint T0				
Variable 1	**Median (IQR)**	**Variable 2**	**Median (IQR)**	**P value, Mann Whitney test**
AHF	15.30 (7.65)	CHF	6.99 (8.70)	**0.018**
AHF	15.30 (7.65)	Obese	9.05 (4.27)	**0.0001**
AHF	15.30 (7.65)	Hypertensive	10.20 (5.05)	**0.003**
AHF	15.30 (7.65)	Non-HF (obese and hypertensive)	9.60 (4.5)	**0.0001**
HF population (AHF+CHF)	14.45 (7.67)	Non-HF (obese and hypertensive)	9.60 (4.5)	**0.001**
CHF	6.99 (8.70)	Obese	9.05 (4.27)	1.000
CHF	6.99 (8.70)	Hypertensive	10.20 (5.05)	0.669
CHF	6.99 (8.70)	Non-HF (obese and hypertensive)	9.60 (4.5)	0.845

AHF = acute HF; CHF= chronic HF; T0=day of admission or day of first exam

Table 24. Correlation between Serum and Salivary Cortisol levels at baseline (T0)			
Population	**Correlation Coefficient**	**R value**	**P value**
All patients, n=97	Pearson	0.206	0.100
AHF patients, n=49	Spearman	0.202	0.293
CHF patients, n=12	Spearman	-0.036	0.939
Obese, n=18	Pearson	0.462	0.072
Hypertensives, n=18	Spearman	0.516	0.071
Non-HF population (Obese and Hypertensive), n=36	Spearman	**0.471**	**0.010**
HF population (AHF+CHF), n=61	Spearman	0.144	0.402

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam.*

Table 25. Correlation between NT-proBNP and Cortisol levels in serum and in saliva.			
Serum NT-proBNP and Serum Cortisol	**Correlation Coefficient**	**R value**	**P value**
All patients at T0, n=97	Spearman	**0.502**	**0.0001**
All patients at T4, n=86	Spearman	**0.540**	**0.0001**
AHF patients at T0, n=49	Spearman	0.315	0.096
AHF patients at T1, n=49	Spearman	0.261	0.171
AHF patients at T4, n=42	Spearman	**0.433**	**0.009**
CHF patients at T0, n=12	Spearman	**0.821**	**0.023**
CHF patients at T4, n=8	Spearman	0.393	0.383
HF population (AHF+CHF) at T0, n=61	Spearman	**0.454**	**0.005**
HF population (AHF+CHF) at T4, n=50	Pearson	**0.453**	**0.003**
Saliva NT-proBNP and Saliva Cortisol	**Correlation Coefficient**	**R value**	**P value**
All patients at T0, n=97	Spearman	-0.080	0.438
All patients at T4, n=86	Spearman	0.114	0.296
AHF patients at T0, n=49	Spearman	-0.075	0.611
AHF patients at T1, n=49	Pearson	0.190	0.202
AHF patients at T4, n=42	Pearson	-0.032	0.840
CHF patients at T0, n=12	Spearman	-0.483	0.112
CHF patients at T4, n=8	Spearman	0.143	0.736
HF population (AHF+CHF) at T0, n=61	Spearman	-0.214	0.098
HF population (AHF+CHF) at T4, n=50	Pearson	0.013	0.929

ALL = whole study population; *AHF = acute HF; CHF = chronic HF, T0 = day of admission or day of first exam; T1 = day of discharge for AHF patients; T4=four week follow-up.*

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