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Salivary lipid mediators: Key indexes of inflammation regulation in heart failure disease

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ABSTRACT

Cardiovascular diseases (CVDs) are the leading cause of premature death and disability in humans and their incidence continues to increase. Oxidative stress and inflammation have been recognized as key pathophysiological factors in cardiovascular events. The targeted modulation of the endogenous mechanisms of inflammation, rather than its simple suppression, will become key in treating chronic inflammatory diseases. A comprehensive characterization of the signalling molecules involved in inflammation, such as endogenous lipid mediators, is thus needed.

Here, we propose a powerful MS-based platform for the simultaneous quantitation of sixty salivary lipid mediators in CVD samples. Saliva, which represents a non-invasive and painless alternative to blood, was collected from patients suffering from acute and chronic heart failure (AHF and CHF, respectively), obesity and hypertension. Of all the patients, those with AHF and hypertension showed higher levels of isoprostanoids, which are key indexes of oxidant insult. Compared to the obese population, AHF patients showed lower levels ($p < 0.02$) of antioxidant omega-3 fatty acids, in line with the "malnutrition-inflammation complex syndrome" typical of HF patients. At hospital admission, AHF patients showed significantly higher levels ($p < 0.001$) of omega-3 DPA and lower levels ($p < 0.04$) of lipoxin B_4 than CHF patients, suggesting a lipid rearrangement typical of the failing heart during acute decompensation. If confirmed, our results highlight the potential use of lipid mediators as predictive markers of re-acutisation episodes, thus providing opportunities for preventive intervention and a reduction in hospitalizations.

1. Introduction

Cardiovascular diseases (CVDs) represent a global burden whose incidence continues to increase [1]. CVDs refer to all the disorders involving the heart and blood vessels, such as coronary heart disease, peripheral vascular disease, and heart failure (HF) [1]. A non-healthy lifestyle, e.g. smoking, bad dietary habits, physical inactivity or chronic stress, are associated with the principal CVDs risk factors, including hypertension, hypercholesterolemia, obesity and diabetes [2]. Oxidative stress and inflammation have gained increasing attention as key pathophysiological factors in cardiovascular events [3], such as HF syndrome. HF is a leading cause of hospitalization and mortality in the West [4], as well as a key comorbidity in cognitive impairment in the elderly [5]. HF disease has been associated with poor quality of life, recurring hospitalizations, and a reduced survival rate [6,7]. The diagnosis of HF is established only after a clear appearance of the clinical symptoms, thus highlighting the importance of understanding and modulating its pathophysiological mechanisms for a successful patient management [8]. Dysfunction of cardiac mitochondria is a hallmark of HF and a leading cause of oxidative stress, which in turn causes myocardial tissue damage and inflammation thus contributing to heart failure progression [9]. Oxidative stress and inflammation are strictly related to each other, both in the acute phase of the disease, e.g., after myocardial infarction, and during chronic cardiac remodelling [9].

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Antioxidants or anti-inflammatory drugs have thus been proposed as supporting therapies for subjects either at high risk or who suffer from established heart failure [9].

The immune-inflammatory response is regulated among cells by macrophages through the expression and secretion of various receptors and soluble molecules, such as cytokines, chemokines and lipid mediators [10]. Lipid mediators, also termed oxylipins, are generated both by omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) through enzymatic (e.g., prostanoids, epoxy, and hydroxy fatty acids) and non-enzymatic (e.g., isoprostanoids) oxidation reactions [11,12]. Oxylipins are implicated not only in the initial phases of inflammation, but also in the precise regulation of its course and resolution. At the very beginning of the inflammatory insult, M1-type macrophages induce the release of pro-inflammatory mediators, such as prostaglandins, thromboxanes, and leukotrienes, leading to the classic signs of inflammation [13]. The production of oxylipins then undergoes a lipid mediator class switching because of the shift from M1-to anti-inflammatory M2-type macrophages. M2-macrophages play a key role in producing pro-resolving mediators (SPMs), including lipoxins, resolvins, protectins and maresins [14], roughly 12 h after the beginning of the inflammatory response. SPMs lower the inflammatory response and even promote its resolution by decreasing the influx of neutrophils to tissues, enhancing non-phlogistic recruitment of monocytes into tissues, as well as decreasing the production of pro-inflammatory mediators [15]. The lack of control of the process and SPM deregulation can lead to a perpetuating inflammatory cycle [16], typical of chronic inflammation. This form of low-grade chronic inflammation often accompanies metabolic disorders, such as obesity and hypertension, and represents a fundamental player in the onset of CVDs [17]. During chronic inflammation, both pro-inflammatory and pro-resolving mediators are present, with specific molecules being over- or under-expressed, according to the different inflammatory diseases and tissues.

All this evidence suggests that several families of bioactive lipid mediators coexist which are differently expressed during the various stages of inflammation, and that these bioactive compounds are needed to work together to regulate the inflammatory response. To date, a fully detailed temporal characterization of these mediators during the different stages of inflammation is still lacking and represents a future challenge in the study of CVDs. In the present work we thus propose a powerful MS-based platform for the quantitation of sixty salivary oxylipins in CVD samples. Saliva, which represents a non-invasive and painless alternative to blood, was collected from patients suffering from acute and chronic heart failure, obesity and hypertension for a comprehensive lipid mediator profiling of one of the most studied CVDs and its main risk factors.

2. Materials and methods

2.1. Chemicals and materials

Acetonitrile and methanol hypergrade for LC-MS LiChrosolv® (purity ≥99.9%), and water for chromatography (LC-MS grade) LiChrosolv® (purity ≥99.9%) used for sample treatment and UHPLC-ESI-MS/MS analysis were from EDM Millipore (Milan, Italy). Commercially available oxylipins (purity ≥99%), 15-F_{2t}-isoprostane, 15-F_{2t}-isoprostane-d4, 15-E_{2t}-isoprostane, 15-E_{2t}-isoprostane-d4, prostaglandin E_2, prostaglandin E_2-d4, prostaglandin D_2, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2, thromboxane B_2, leukotriene B_4, lipoxin A_4, lipoxin A_4-d5, lipoxin B_4, resolvin E_1, resolvin D_1, resolvin D_1-d5, resolvin D_2, resolvin D_3, resolvin D_4, resolvin D_5, 17(*R*)-Resolvin-D_1, 17(*R*)-Resolvin-D_1-d5, 17(*R*)-Resolvin-D_3, 17(*R*)-Resolvin-D_4, neuroprotectin D_1, protectin DX, maresin-1, maresin-1-d5, 7-epi-maresin-1, maresin-2, 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, 15-HETE, 20-HETE, 20-HETE-d6, 8,9-DiHETE, 11,12-DiHETE, 14,15-DiHETE, 13-hydroxy-9Z,11E-octadecadienoic acid (HODE), 8,9-epoxyeicosatrienoic acid (EET), 11,12-EET, 14,15-EET, 14,15-EET-d11, adrenic acid (AdA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA) docosahexanoic acid (DHA), arachidonic acid (AA), docosapentaenoic acid (DPA), linoleic acid (LA) and LA-d4 were from Cayman Chemical (Michigan, USA). Non-commercially available oxylipins, i.e. 5-F_{2t}-isoprostane, 5-*epi*-5-F_{2t}-isoprostane, 8-F_{3t}-isoprostane, 8-*epi*-8-F_{3t}-isoprostane, 18-F_{3t}-isoprostane, 20-F_{4t}-neuroprostane, 20-*epi*-20-F_{4t}-neuroprostane, 10-F_{4t}-neuroprostane-d4, 10-*epi*-10-F_{4t}-neuroprostane-d4, 14(*R,S*)-14-F_{4t}-neuroprostane, 14(*R,S*)-14-F_{3t}-neuroprostane, 4(*R,S*)-4-F_{4t}-neuroprostane, C21-15-F_{2t}-isoprostane, tetranor-NPD_1, dinor-NPD_1, 7(*R,S*)-ST-Δ^8-11-dihomo-isofuran, ent-7(*R,S*)-7-F_{2t}-dihomo-isoprostane, 17-F_{2t}-dihomo-isoprostane, linotrin, di$H_{n\text{-}3}$-DPA, di$H_{n\text{-}6}$-DPA and diH-AdA were synthesized at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France), according to procedures reported elsewhere [18–20].

Sterile polypropylene containers were from Eppendorf (Milan, Italy), whereas Phenex™-RC syringe filters (0.2 μm regenerate cellulose, 4 mm diameter) were from Phenomenex (California, USA). A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and a Hermle Z-326 K Centrifuge, (Wehingen, Germany) were used for sample vortex-mixing and centrifugation, respectively. The removable needle micro-extraction by packed sorbent (MEPS) 250 μL syringe for HTA 300APlus (Thermo Scientific & Varian 8400 systems) and MEPS silica-C18 Barrel Insert and Needles (BINs) were purchased from SGE Analytical Science (Melbourne, Australia). The automated HT4000 Series Sample Prep workstation was purchased from HTA S.R.L. (Brescia, Italy).

2.2. Preparation of standard solutions and quality control samples

Stock solutions of commercially available oxylipins (100–1000 μg/mL in organic solvent [see product insert from Cayman Chemicals]), as well as those synthesized at IBMM in Montpellier, France (1000 μg/mL in methanol), were stored at −80 °C and used for the preparation of intermediate standard mixtures containing all the 60 oxylipins in methanol:acetonitrile (1:1, *v/v*) mixed solvent.

Working standard solutions of the 60 oxylipins were prepared by diluting intermediate stock solutions with LC-MS water (stored at 4 °C for up to one month). A mixture of deuterated internal standards (ISs, 15-F_{2t}-isoprostane-d4, 15-E_{2t}-isoprostane-d4, 10-F_{4t}-neuroprostane-d4, 10-*epi*-10-F_{4t}-neuroprostane-d4, C21-15-F_{2t}-isoprostane, prostaglandin E_2-d4, lipoxin A_4-d5, resolvin D_1-d5, 17(*R*)-Resolvin-D_1-d5, maresin-1-d5, 20-HETE-d6, 14,15-EET-d11 and LA-d4) was prepared at 20 ng/mL through sequential dilutions of the stock solutions (100 μg/mL in organic solvent [see product insert from Cayman Chemicals]) with methanol:acetonitrile (1:1, *v/v*). Working standard solutions were used to obtain five-point calibration curves (see concentration levels in Table 1). These solutions, to which 20 μL of the ISs mixture (20 ng/mL) were added, were then subjected to the MEPS procedure and analysed by UHPLC-ESI-MS/MS.

Saline solutions of copper sulphate pentahydrate (10% w/v) and sodium tungstate dihydrate (12% w/v) were prepared by dissolving the required amount of each salt in LC-MS water.

Pooled saliva samples were obtained by mixing known aliquots of stimulated saliva samples collected from healthy volunteers by Salivette® polyester swab (Sarstedt, Nümbrecht, Germany). This pooled sample was spiked with a known amount of the intermediate stock solutions to obtain quality control samples (QCs) at low, medium, high levels (i.e. levels 1, 3, and 5 of the calibration curve), which were used for method validation. The volume of the spiked standard solution never exceeded 10% of the total saliva sample volume.

2.3. Saliva sample extraction and analysis by MEPS-UHPLC-MS/MS

Sample treatment and analysis were performed by extending our analytical protocol previously developed in plasma [21] to the less conventional saliva matrix. Our protocol successfully couples the new

Table 1
Full list of quantified oxylipins and relevant analytical parameters i.e., calibration curve, limit of detection, intra-assay and inter-assay recovery, intra-assay and inter-assay precision.

Compound	Calibration levels (pg/mL, *ng/mL)	Slope ± s.d (R^2)	LOD (pg/mL, *ng/mL)	Intra-assay recovery % (RSD)[a]	Inter-assay recovery % (RSD)[b]
PUFAs					
AA	1; 5; 10; 25; 50*	110 ± 10 (0.98)	3*	101(5)	91(12)
DHA		310 ± 30 (0.98)	7*	101(7)	92(11)
DPA_{n-3}		116 ± 6 (1.00)	10*	100(8)	100(12)
EPA		95 ± 7 (0.99)	1*	100(9)	96(11)
Isoprostanoids (IsoPs, NeuroPs, dihomo-isoPs), isofurans (IsoFs) and prostanoids (prostaglandins (PGs), leukotrienes (LEU), thromboxanes (TX))					
5-F_{2t}-IsoP	0.05; 0.10; 0.25; 0.50; 1	0.19 ± 0.02 (1.00)◊	10	101(3)	111(8)
5-*epi*-5-F_{2t}-IsoP		0.15 ± 0.01 (1.00)◊	15	100(7)	110(10)
15-F_{2t}-IsoP		1.1 ± 0.1 (1.00)◊	10	102(8)	109(9)
8-F_{3t}-IsoP		1.08 ± 0.13 (1.00)	10	101(4)	111(8)
8-*epi*-8-F_{3t}-IsoP		1.15 ± 0.03 (1.00)	15	101(7)	107(9)
18-F_{3t}-IsoP		0.17 ± 0.03 (1.00)	5	103(3)	96(11)
15-E_{2t}-IsoP		14 ± 1 (0.98)◊	7	101(8)	93(12)
4(*R,S*)-4-F_{4t}-NeuroP		0.51 ± 0.06 (1.00)	70	100(3)	105(7)
20-F_{4t}-NeuroP		0.095 ± 0.003 (0.99)	20	101(3)	98(4)
20-*epi*-20-F_{4t}-NeuroP		0.07 ± 0.01 (0.99)◊	610	100(11)	90(15)
14(*R,S*)-14-F_{4t}-NeuroP		0.076 ± 0.003 (0.99)	250	100(10)	107(9)
14(*R,S*)-14-F_{3t}-NeuroP		0.54 ± 0.05 (1.00)	15	88(11)	95(10)
ent-7(*R,S*)-7-F_{2t}-dihomo-isoP		0.52 ± 0.02 (0.99)◊	5	101(9)	117(13)
17-F_{2t}-dihomo-isoP		0.78 ± 0.09 (0.99)	70	101(7)	106(6)
7(*R,S*)-ST-Δ^8-11-dihomo-isoF		0.15 ± 0.01 (1.00)◊	20	102(3)	94(12)
PGE_2	0.1; 0.5; 1; 2.5; 5	4.9 ± 0.7 (1.00)◊	4	100(4)	100(12)
PGD_2		1.07 ± 0.09 (1.00)◊	20	101(9)	106(18)
15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2		1.1 ± 0.1 (0.98)		100(9)	91(6)
LEU-B_4	0.5; 5; 10; 25; 50	4.2 ± 0.3 (0.98)	10	97(3)	94(5)
TX-B_2		0.23 ± 0.02 (0.99)◊	70	98(6)	103(13)
Hydroxy/dihydroxy-PUFAs					
5-HETE	0.5; 5; 10; 25; 50	31 ± 4 (1.00)◊	17	100(8)	99(9)
12-HETE		26 ± 3 (0.99)◊	83	97(9)	85(15)
15-HETE		110 ± 10 (0.97)	7	104(6)	91(7)
20-HETE		3.5 ± 0.4 (0.98)	15	101(12)	106(17)
13-HODE	2.5; 25; 50; 125; 250	28 ± 5 (0.97)	1*	103(10)	115(14)
8,9-DiHETE	0.1; 0.5; 5; 10; 25	9.3 ± 0.9 (0.96)◊	11	103(6)	92(11)
11,12-DiHETE		16 ± 1 (0.97)	5	102(11)	114(13)
14,15-DiHETE		11 ± 1 (1.00)◊	10	101(6)	115(12)
Epoxy-PUFAs					
8(9)-EET	0.5; 5; 10; 25; 50	0.67 ± 0.03 (0.97)◊	180	110(10)	102(12)
11(12)-EET		4.3 ± 0.6 (0.96)◊	50	101(4)	115(8)
14(15)-EET		4.3 ± 0.4 (0.96)◊	320	111(6)	106(3)
Pro-resolving (lipoxins, resolvins, maresins, protectins)					
Lipoxin-A_4	0.1; 0.5; 2.5; 5; 20	80 ± 10 (0.99)◊	830	105(6)	118(15)
Lipoxin-B_4		1.29 ± 0.09 (0.97)◊	10	109(6)	113(12)
Resolvin-D_1		1.00 ± 0.08 (0.99)	4	101(7)	110(12)
Resolvin-D_2		0.15 ± 0.01 (1.00)◊	40	100(7)	97(18)
Resolvin-D_3		0.65 ± 0.03 (1.00)◊	30	100(11)	95(10)
Resolvin-D_4		0.41 ± 0.07 (0.95)◊	55	97(11)	97(13)
Resolvin-D_5		4.7 ± 0.5 (0.99)	8	102(7)	119(16)
17(R)-Resolvin-D_1		1.5 ± 0.1 (0.98)	20	101(7)	109(10)
17(R)-Resolvin-D_3		1.02 ± 0.07 (1.00)◊	5	106(10)	108(13)
17(R)-Resolvin-D_4		0.41 ± 0.07 (0.95)◊	770	97(11)	97(13)

(*continued on next page*)

Table 1 (*continued*)

Compound	Calibration levels (pg/mL, *ng/mL)	Slope ± s.d (R^2)	LOD (pg/mL, *ng/mL)	Intra-assay recovery % (RSD)[a]	Inter-assay recovery % (RSD)[b]
Resolvin-E_1		1.0 ± 0.2 (0.99)	80	87(13)	91(9)
Maresin-1		0.72 ± 0.07 (0.98)	130	103(6)	114(9)
7-epi-maresin-1		0.95 ± 0.05 (1.00)◊	5	101(2)	116(7)
Maresin-2		1.95 ± 0.07 (1.00)◊	10	100(9)	104(13)
Neuroprotectin D_1		3.8 ± 0.2 (0.98)	15	102(5)	107(8)
dinor-Neuroprotectin D_1		14 ± 2 (1.00)◊	5	101(6)	114(8)
Tetranor-Neuroprotectin D_1		1.36 ± 0.09 (1.00)◊	40	101(7)	93(13)
Protectin DX		7.7 ± 0.5 (0.98)	11	101(10)	114(11)
diH-AdA		1.9 ± 0.1 (0.98)	50	102(7)	116(10)
$diH_{n\text{-}3}$-DPA		2.4 ± 0.1 (0.97)	50	101(8)	109(13)
$diH_{n\text{-}6}$-DPA		11.5 ± 0.8 (1.00)◊	5	101(3)	103(8)
Linotrin		14 ± 1 (0.99)◊	70	103(5)	109(8)

◊ These slopes refer to calibration curves prepared in the saliva matrix due to the statistically significant ($p < 0.05$) matrix effect.

[a] Calculated from three replicates at low, medium, high (i.e. levels 1, 3, and 5 of the calibration curve) concentration values.

[b] Calculated from three replicates at low, medium, high (i.e. levels 1, 3, and 5 of the calibration curve) concentration values in three days.

micro-extraction by packed sorbent (MEPS) technique to liquid chromatography tandem mass spectrometry for the straightforward analysis of oxylipins in various biofluids [21–23].

Briefly, an aliquot of saliva sample (500 μL) to which 20 μL of the ISs mixture (20 ng/mL) were added, was then treated by the sequential addition of salts (i.e., 250 μL of $CuSO_4{\cdot}5\ H_2O$ 10% w/v and 250 μL of $Na_2WO_4{\cdot}2\ H_2O$ 12% w/v) and acetonitrile (500 μL) for protein precipitation. After vortex-mixing (2000 rpm for 1 min), centrifugation (7000 rpm for 5 min), and filtration at 0.2 μm, the supernatant was diluted (1:5 *v/v*) with water and subjected to a clean-up procedure by the MEPS technique. The MEPS C18 cartridge was activated by drawing and discharging 100 μL of methanol (3 × 100 μL) three times, which was then conditioned with 3 × 100 μL of water at 0.6 mL/min. The diluted supernatant (3000 μL) was loaded up and down twelve times at 0.3 mL/min by discarding it. The cartridge was then washed with 100 μL of a water:methanol (95:5 *v/v*) mixture at 0.6 mL/min to remove potential interferences. Analytes were eluted with 50 μL of methanol at 0.3 mL/min and then injected into the UHPLC-MS/MS instrument.

The instrument consisted of an Agilent 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer, which was equipped with a Jet Stream electrospray (ESI) ionization source (Agilent Technologies, USA). The chromatographic separation was achieved at 0.7 mL/min using a Polaris 3 C18-A column (50 × 4.6 mm, 3 μm, Agilent Technologies, USA) and a gradient elution with a mobile phase consisting of 0.1% aqueous formic acid (A) and 50:50 *v/v* methanol: acetonitrile (B). The multisampler and the column compartment were set at 4 and 25 °C, respectively. The injection volume was 20 μL. The Agilent 6495 Triple Quadrupole mass spectrometer detector operated in ESI negative ionization mode and performed multiple reaction monitoring with unit mass resolution. Mass spectrometer control, data acquisition and data analysis were performed with MassHunter Workstation software (B.07.00). Each analyte was detected using two specific MRM transitions. The most abundant one was used for the quantification (Q) of the target compound whereas the other was used for its identification (q). A deviation ≤ ± 0.10 min of the expected retention time compared to working standard solutions and a qualifier/quantifier (q/Q) ratio within 20% of the ratio measured in working standard mixtures were required for the identification of the analyte in the saliva. Detailed chromatographic parameters, and ESI and MRM operating conditions are shown elsewhere [21].

2.4. MEPS-UHPLC-MS/MS method validation

The analytical method was validated according to the IUPAC guidelines [24]. This included the evaluation of the limits of detection (LODs), calibration curves, matrix effect, recovery, and intra-day and inter-day precision. LODs were calculated as three times the standard deviation (s.d.) of the "low level spiked blank". Five-point external and internal calibration curves were obtained by analyzing in triplicate working standard solutions and pooled saliva samples spiked with known amounts of standards, respectively. As well as the spiked saliva samples, 20 μL of the ISs solution (20 ng/mL) were added to these solutions, which were then subjected to the MEPS procedure and analysed by UHPLC-ESI-MS/MS.

The analyte (a) to internal standard (IS) peak area ratios (Y, A_a/A_{IS}) were plotted versus the corresponding concentration ratios (X, C_a/C_{IS}). Calibration curves (Y = mX) for all the analytes were fitted by the Deming regression, which considers measurement errors for both dependent and independent variables. The matrix effect was evaluated by comparing, at a confidence level of 95%, the slopes of the calibration curves obtained with working solutions (external) and spiked saliva samples (internal). In case of a matrix effect, internal calibration curves were used for analyte quantitation.

Recovery was assessed by analysing a pooled saliva sample spiked at three concentration levels (low, mid, and high) in triplicate (n = 3 at each concentration level) within the same day and on three consecutive days. The analyte recovery was calculated as the percentage ratio of the difference between the average analyte concentration measured in the spiked and non-spiked samples to the expected concentration. Intra- and inter-day precision were expressed as the relative standard deviation (RSD) of measurements performed on the spiked samples on a single day and on three consecutive days, respectively.

2.5. Study design

The study was carried out within the framework of the KARDIATOOL project ('An integrated PoC solution for diagnosis and therapy monitoring of heart failure patients', Grant agreement N°: 768,686) and was approved (protocol number: 54,764) by the local ethics committee (Area Vasta Nord-Ovest, CEAVNO). The study was conducted in accordance with the 1964 Declaration of Helsinki and all subjects gave written informed consent after receiving appropriate information on the protocol. The study population consisted of patients admitted to hospital with acute heart failure (AHF, n = 17), patients with symptoms of HF and known HF, i.e. chronic stable HF (CHF, n = 7), patients with symptoms without HF, but with obesity (n = 10) and patients with symptoms without HF, but with hypertension (n = 7). Each AHF subject was monitored at hospital admission (due to a re-cutisation episode), at hospital discharge and one month later during the follow-up visit. Patients with hypertension and obesity were monitored twice a month.

The inclusion criteria for the present study were: patients with a diagnosis of AHF requiring hospital admission or outpatient intravenous diuretics; patients with known stable heart failure; patients with a diagnosis of heart failure (with reduced or preserved ejection fraction) and any underlying aetiology, according to the European Society of Cardiology guidelines 2016 [25]; patients with obesity (with a body mass index greater than 30 kg/m^2) and HF symptoms but no evidence of ventricular dysfunction on echocardiography or raised natriuretic peptides; and patients with hypertension defined as systolic blood pressure ≥140 mmHg ± diastolic blood pressure ≥90 mmHg or >1 month use of antihypertensive medications, and HF symptoms but no evidence of ventricular dysfunction on echocardiography or raised natriuretic peptides.

The exclusion criteria included patients aged under 18 years of age, patients unable or unwilling to give informed consent, patients with cognitive disorders, mental disorders or psychiatric disorders, with alcohol abuse 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, patients with severe co-morbidities such as: severe COPD (e.g., patient is on oxygen therapy or on nebulizers), uncontrolled dysthyroidism, i.e. TSH <0.03 or >7, decompensated diabetes, 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, i.e. eGFR <30 ml/min/1.73 m^2.

For each patient, information on demographics, clinical history, co-morbidities, previous therapies and physical status were collected. In the case of a HF re-acutisation episode and the subsequent hospitalization, patients were treated in accordance with the recommendations for attending physicians from the ESC guidelines [25].

Saliva sampling was performed in accordance with the procedures already developed in our laboratory and reported elsewhere [26,27]. Briefly, stimulated saliva samples were collected by asking the patients to roll a Salivette polyester swab in the mouth for 2 min. Circadian cycle effects and interference from fasting were minimized by always collecting samples in the morning (10.00–12.00 a.m.). After sample collection, salivary pH was measured using a narrow range pH paper. The salivary flow rate (mL/min) was calculated from the ratio between the weight difference (g) of the sampling device before and after sampling and the collection time (min), considering the density of sample equal to 1 g/mL [28]. The sample was recovered by centrifuging the swab at 7000 rpm and 4 °C for 5 min and was stored in a polypropylene tube at −80 °C until use.

2.6. Statistical analysis

GraphPad Prism (v. 9.0) and MetaboAnalyst v. 5.0 (Wishart Research group, The Metabolomics Innovation Centre (TMIC), University of Alberta, Canada (http://www.metaboanalyst.ca)) were used for the statistical data analysis. The statistical relationship between variables was examined by Pearson's correlation. Univariate (non-parametric Wilcoxon and Kruskal-Wallis test) and multivariate analysis ((PCA and cluster analysis [29]) were performed on the dataset. The original data were pre-processed (data integrity and missing value check) and normalized (i.e., data transformation and data scaling) before the statistical analysis. Continuous variables with a normal distribution were reported as mean ± standard deviation, whereas variables with skewed distribution were described by the median with lower (25th percentile) and upper (75th percentile) quartiles.

3. Results and discussion

3.1. Optimization of saliva sample extraction

Sample pre-treatment is a critical step in the analysis of biofluids [30]. The mucins and other proteins in saliva prevent its direct loading onto the MEPS cartridge, thus a preliminary sample treatment step prior to MEPS extraction is required to minimize the saliva viscosity and extend the lifetime of the cartridge. Here, we compared the analytical performances of a combined addition of salts and acetonitrile (method A [21]) and a simple and straightforward 6-fold saliva dilution with water (method B [31]). Method A entailed the consecutive addition of 250 μL of $CuSO_4$·5 H_2O 10% *w/v* and 250 μL of Na_2WO_4·2 H_2O 12% *w/v* and 500 μL of acetonitrile to 500 μL of spiked saliva samples (medium concentration level). After vortex-mixing (2000 rpm for 1 min) and filtration at 0.2 μm, the resulting supernatant was then diluted (1:5 *v/v*) with water. In method B, the spiked saliva sample was directly diluted with water (1:5 *v/v*) and filtered at 0.2 μm. Each procedure was performed in quintuplicate. For both methods the diluted supernatant/sample (3000 μL) was loaded up and discharged 12 times by MEPS and the target analytes were eluted with 50 μL of methanol, maintaining the same sample-to-elution solvent volume ratio across all the experiments.

Both procedures showed a comparable analytical performance in terms of analyte peak area and extraction efficiency. The analyte recovery was almost quantitative for both the combined addition of salts and acetonitrile (87–111%, RSD <15%) and the 6-fold water dilution (75–119%, RSD <15%).

In the case of water dilution (method B), a pronounced increase in the background noise in the chromatographic profile was observed after five consecutive UHPLC-MS/MS injections. This occurred particularly within the timeframe starting from minute ten up to the end of the chromatographic run and corresponding to the acquisition of pro-resolving mediators, epoxy and hydroxy-fatty acids, and PUFAs signals. A laborious cleaning of both column and ESI source would thus be needed to restore the chromatographic baseline after a typical batch sequence (10–15 samples). The combined addition of salts and acetonitrile was thus preferred to the dilution of the saliva sample prior to MEPS-UHPLC-MS/MS analysis. Compared to the time consuming and multistage procedures reported elsewhere [32], our approach consists of a *single-step* saliva clean-up and analyte enrichment (10-fold) in about 30 min using a reduced amount of organic solvents (i.e. 50-100 μL), as well as avoiding any drying step before injection.

3.2. Analytical figures of merit

LOD values ranged between 5 and 800 pg/mL for oxylipins and 30–1000 ng/mL for PUFAs, which in most of cases were much lower than those reported in the literature [33–35], and which were suitable for a reliable determination of oxylipins in saliva [32]. All the analytes showed adequate linearity with R^2 between 0.96 and 1.00 within the tested concentration range. Recoveries were not significantly different ($p > 0.05$) across the concentration levels tested and were satisfactory for most of the analytes when performing both intra- (overall range: 88–110%) and inter-day (overall range: 85–119%) experiments. Intra- and inter-day precisions were always lower than or equal to 20%. The slopes of the calibration curves obtained with working solutions (external curve) and spiked samples (internal curve) were not significantly different at a confidence level of 95% for half of the analytes (see Table 1), suggesting the absence of any measurable matrix effect. The difference was slightly significant ($p < 0.05$) for thirty out of sixty oxylipins (see Table 1), for which internal calibration curves were used for analyte quantitation.

Table 1 reports the mean slopes and standard deviations (n = 3) of the five-point calibration curves, LOD values and intra- and inter-day recovery, with the corresponding RSD.

3.3. Preliminary data on heart failure, hypertensive, and obese patients

Within the framework of the clinical pilot study, AHF (n = 17) and CHF (n = 7), hypertensive (n = 7) and obese (n = 10) patients were enrolled. AHF subjects were monitored at hospital admission (due to a

re-cutisation episode), at hospital discharge and one month later during the follow-up visit. Hypertensive and obese subjects were monitored twice a month, for a total of 91 samples collected and analysed. For these patients, clinical parameters and oxylipin levels did not change significantly ($p > 0.05$) within that month thus samples collected at those collection points were considered as biological replicates of the same population. Demographic and clinical data of both AHF and CHF, hypertensive and obese patients are reported in Table 2.

The demographics were generally similar between the different groups, except in terms of the prevalence of females in obese patients. Pharmacological therapy was almost comparable among the different pathologies except for the high prevalence of beta blockers (80–100%), diuretics (80–100%) and anticoagulants (50–60%) in HF patients, and calcium channel blockers (57%) in patients with hypertension. Most HF patients showed a high incidence of coronary artery disease (76%) as well as diabetes mellitus (47%) in the case of acute decompensation. Oxylipin levels were not significantly different ($p > 0.05$) when patients were grouped according to those comorbidities. AHF patients were characterized by significantly higher levels of urea ($p < 0.001$) and creatinine ($p = 0.002$), and lower levels of potassium ($p = 0.006$), lower full blood count ($p < 0.001$) and iron ($p = 0.001$) content compared with CHF, hypertensive and obese patients. As expected, AHF patients showed very high levels of NT-proBNP which differed significantly ($p < 0.001$) hypertensive and obese patients. When patients were grouped in one of four categories in the NYHA functional class, lipoxin-B_4, DHA and omega-3 DPA content differed significantly among the four classes ($p = 0.02$).

Oxylipins were quantified in the saliva samples collected from all the participants. Thirty-four out of sixty oxylipins were excluded from the dataset as the concentrations were below the limit of quantification for more than 50% of the samples. This may be expected since only a fraction of the free oxylipins circulating in blood may be transferred in oral fluid depending on the physical and chemical properties of the compound (e.g. molecular size, pKa and lipid solubility [36]). This aspect will be investigated further in the near future.

The data matrix used for the subsequent statistical analysis therefore consisted of 87 samples and 26 features (oxylipins). Fig. S1 shows the chromatographic profiles for the 26 oxylipins, whereas their concentration levels [median (min-max)] are reported in Table S1. Data were analysed by PCA in order to summarize and visualize the information content of our data matrix (Fig. 1).

In the PCA, variables contributing similar information were grouped together, that is, they were correlated. When the variables correlated negatively, they were positioned on opposite sides of the plot origin. Observations characterized by a high value of a specific variable were located close to this value in the 3D-space. In our case, the three lowest-order principal components accounted for a total explained variance of approximately 36%. Samples were spread throughout the space along both PC1 and PC3 directions. Conversely, PC2 scores contributed to the slight separation between i) AHF patients, and ii) CHF as well as obese patients. An up-to-down switch of the oxylipin profile was observed when moving from an acute to chronic inflammation status, i.e. from AHF to both CHF and obesity [37]. Hypertensive samples were spread halfway the samples of the two sub-groups. Samples from AHF patients (admission discharge, follow-up) were located in the upper part (dotted red circle, Fig. 2A), thus highlighting an over-expression of different families of isoprostanes (e.g. F_2-isoprostanes, F_2-dihomo-isoprostanes (Fig. 2B)). These mediators are indexes of oxidative damage and are typically released from the very beginning of an oxidant/inflammatory injury. Otherwise, samples from both CHF and obese subjects were located at the bottom of PC2 (dotted blue circle, Fig. 2A) in the direction of omega-3 PUFAs and pro-resolving mediators (Fig. 2B), thus suggesting low-grade and late-stage persistent inflammation.

Fig. 2 shows the median omega-3 PUFA values (i.e., EPA (20:5), DPA (22:5) and DHA (22:6)) as well as AA-derived pro-resolving lipoxin-B_4 which varied significantly ($p < 0.05$) among groups. The obese population showed significantly higher levels [median (lower and upper quartiles)] of EPA [30(20–55) *vs* 14(10–18) ng/mL, $p < 0.02$], DHA [40 (32–70) *vs* 21(14–25) ng/mL, $p < 0.02$] and lipoxin-B_4 [75(32–123) *vs* 16(10–44) pg/mL, $p < 0.005$], compared to AHF patients monitored during hospitalization. AHF patients at admission presented higher levels of omega-3 DPA [12(8–21) *vs* 3(3–4) ng/mL, $p = 0.001$] and lower levels of lipoxin-B_4 [16(10–44) *vs* 39(30–65) pg/mL, $p = 0.044$], as opposed to CHF subjects. Interestingly, the lipoxin-B_4 concentration in the AHF population showed a notable increase (6-fold) over time, one month later (follow-up measurement) reaching comparable levels [50 (24–85) pg/mL] to those of the CHF [39(30–65) pg/mL] and hypertensive [47(29–66) pg/mL] group (Fig. 2).

None of the investigated oxylipin levels varied significantly ($p > 0.05$) between admission and discharge of AHF subjects. The longitudinal monitoring of each subject was hypothesized to be more informative since patients would act as their own controls [38,39]. Fig. 3 shows the changes in oxylipin concentrations for each AHF patient from admission to discharge, normalized with respect to the admission level. HF patients had a notable decrease (40%) of 15-E_{2t}-IsoP concentration over time (Fig. 3), thus showing an incremental ability to differentiate between the admission (acute phase) and discharge point.

Bioactive lipid mediators belong to a very complex network of chemicals whose regulation is still not completely understood. A comprehensive study of their temporal production and the bioactive role exerted within the different phases of the inflammatory cascade is challenging. In the present study, we observed the change in the oxylipin pattern that was strictly related to the inflammatory phase expressed in the different populations. The oxylipin profile in AHF patients was rich in isoprostanes, such as 15-F_{2t}-IsoP, 5-F_{2t}-IsoP, and 15-E_{2t}-IsoP, which represent well-known markers of oxidative stress. In a failing heart, excess mitochondrial ROS, as well as the inability to supply energy, are the main causes of mitochondrial dysfunction and contractile failure found in both humans and animal models [40]. Mitochondrial damage itself causes further increase in ROS production and more severe injury, revealing the key role of oxidative stress in the onset and progression of CVDs [41]. Isoprostanes are reliable markers in assessing the oxidative stress status [42]. Apart from being indexes of oxidative damage, these mediators exert various bioactive roles in vivo, such as vasoactivity, stimulation of both mitogenesis and atherogenesis [43,44] F_2-isoprostanes have been found to play a significant role in the progression of HF from asymptomatic to symptomatic and in the deterioration of the functional capacity [45]. The levels of these lipid mediators were found to correlate consistently to the severity of HF and can thus be tested as long-term prognostic markers [46]. Interestingly, the urinary 15-F_{2t}-isoprostane concentration has been found to be significantly correlated with the concentration of plasma BNP and was found to decrease gradually in proportion to the severity of chronic HF during hospitalization [8,47,48]. In the present work, none of the investigated oxylipins changed significantly during hospitalization, i.e. from admission to discharge, except for 15-E_{2t}-isoprostane. Compared with the F_2-isoprostanes, E_2-isoprostanes are more potent vasoactive mediators [43]. 15-E_{2t}-IsoP is more potent than 15-F_{2t}-IsoP in systemic and pulmonary vessels, showing vasoconstrictor as well as vasodilator activity through thromboxane (TP)- and prostaglandin (EP)-receptors, respectively [49]. The high levels of 15-E_{2t}-IsoP at hospital admission in AHF subjects suggest a pronounced oxidative insult occurring at the very beginning of the decompensation episode, which faded over the course of the hospitalization. However, the lack of sufficient data available on the production of E_2-isoprostanes in cardiovascular disorders, makes it almost impossible to draw further conclusions regarding the potential bioactive role exerted in the failing heart. As with AHF patients, hypertensive subjects showed an over-expression of isoprostanes and, thus, their samples were superimposed in the score plot of the PCA (Fig. 1). Recent studies have indicated oxidative stress, and in particular 15-F_{2t}-isoprostane levels, as a risk factor for blood pressure elevation in both severe and essential hypertensive patients [50].

Table 2
Demographic and clinical characteristics of enrolled patients (n = 41) at the hospital admission (t_0), discharge (t_d) and follow-up 1 month later ($T_{f\text{-}up}$). Data are shown as median [interquartile range, calculated as 75th - 25th percentiles]. Bold values denote statistical significance at $p < 0.05$ level (non-parametric Kruskal-Wallis test).

	AHF (n = 17)			CHF (n = 7)		Hypertensive (n = 7)		Obese (n = 10)		p
Age, years (mean ± SD)	74 ± 9			70 ± 6		70 ± 10		60 ± 10		0.095
Sex (M:F)	13:4			6:1		3:4		3:7		
Comorbidities										
Diabetes (n (%))	8 (47%)			1 (14%)		1 (14%)		0 (0%)		
Hypertension (n (%))	10 (59%)			4 (57%)		7 (100%)		5 (50%)		
Hypercholesterolemia (n (%))	8 (47%)			3 (43%)		5 (71%)		6 (60%)		
Coronary Artery Disease (n (%))	13 (76%)			4 (57%)		0 (0%)		1 (10%)		
Drugs										
ACE inhibitor (n; %)	4 (24%)			5 (71%)		2 (29%)		4 (40%)		
Beta blocker (n; %)	15 (88%)			7 (100%)		3 (43%)		4 (40%)		
Loop diuretic (n; %)	17 (100%)			6 (86%)		0 (0%)		2 (20%)		
Antiplatelet (n; %)	6 (35%)			1 (14%)		3 (43%)		2 (20%)		
Anticoagulant (n; %)	11 (65%)			4 (57%)		0 (0%)		1 (10%)		
Statin (n; %)	9 (53%)			5 (71%)		3 (43%)		2 (20%)		
Calcium channel blocker (n; %)	2 (12%)			0 (0%)		4 (57%)		0 (0%)		
Alpha blocker (n; %)	2 (12%)			0 (0%)		1 (14%)		0 (0%)		
Clinical characteristics										
NYHA class	**t_0**	**t_d**	**$T_{f\text{-}up}$**[a]	**t_0**	**$T_{f\text{-}up}$**	**t_0**	**$T_{f\text{-}up}$**	**t_0**	**$T_{f\text{-}up}$**	
NYHA I (n (%))	0 (0%)	2 (12%)	3 (33%)	3 (43%)	2 (29%)	1 (14%)	1 (14%)	0 (0%)	1 (10%)	
NYHA II (n (%))	3 (18%)	8 (47%)	5 (56%)	4 (57%)	5 (71%)	6 (86%)	6 (86%)	10 (100%)	9 (90%)	
NYHA III (n (%))	11 (65%)	6 (35%)	1 (11%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
NYHA IV (n (%))	3 (18%)	1 (6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
NT-proBNP (from serum) [pg/mL]	4503 [3025–8896]	2358 [1669–5244]	4541 [3200–5078]	755 [307–1679]	452 [212–2052]	76 [57–118]	48 [27–63]	55 [38–80]	44 [31–97]	**<0.001**
Full Blood Count [g/dL]	12.5 [11.5–12.9]	12.0 [10.7–13.0]	11.5 [10.8–12.2]	14.0 [13.2–15.0]	14.1 [13.8–15.5]	14.0 [12.9–14.4]	13.8 [13.5–14.1]	13.4 [13.0–13.9]	13.9 [13.7–14.4]	**<0.001**
Iron (Fe) [μg/dL]	49 [43–57]	48 [44–55]	60 [46–71]	95 [85–104]	81 [79–94]	79 [77–102]	93 [89–98]	80 [62–91]	72 [68–97]	**0.001**
Ferritin [ng/mL]	115 [77–218]	101 [86–149]	76 [40–180]	134 [37–265]	215 [68–241]	104 [94–187]	162 [88–266]	130 [56–150]	175 [108–183]	0.989
Urea [mg/dL]	65 [60–78]	75 [53–91]	57 [49–98]	47 [43–50]	51 [47–58]	40 [35–45]	40 [37–46]	49 [37–51]	35 [33–40]	**<0.001**
Creatinine [mg/dL]	1.5 [1.3–1.7]	1.4 [1.1–1.6]	1.6 [1.0–1.6]	1.0 [0.7–1.1]	0.9 [0.8–1.2]	0.8 [0.7–1.1]	1.1 [1.0–1.1]	0.9 [0.8–1.0]	0.8 [0.7–1.0]	**0.002**
Sodium [mmol/L]	139 [139–141]	142 [141–143]	141 [138–142]	141 [141–142]	140 [140–142]	142 [140–142]	140 [139–141]	140 [139–142]	140 [139–141]	0.421
Potassium [mmol/L]	3.8 [3.8–4.0]	3.8 [3.7–4.0]	4.0 [3.8–4.2]	4.4 [4.1–4.5]	4.4 [4.3–4.8]	4.0 [3.8–4.3]	4.5 [4.1–4.8]	4.4 [4.4–4.7]	4.3 [4.1–4.6]	**0.006**

[a] Only 9 out of 17 patients were monitored at the follow-up visit after one month.

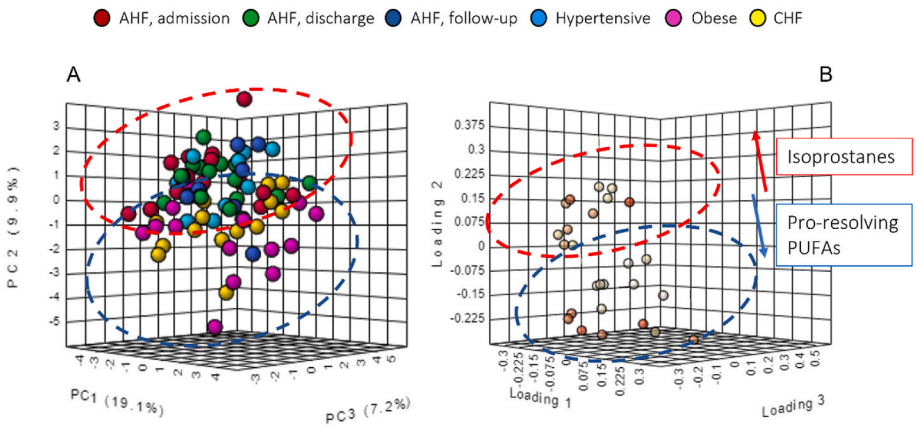

Fig. 1. 3D principal component analysis (PC1 (19.1%), PC2 (9.9%), PC3 (7.2%)) based on the 26 features detected in more than 50% of the samples: score plot (A) and loading plot (B). Score plot: saliva samples collected from AHF patients at hospital admission (red), discharge (green) and follow-up one month later (blue), hypertensive (sky blue), obese (pink), and CHF (yellow) patients. The original data were normalized (i.e., logarithmic transformation and Pareto scaling) before the statistical analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

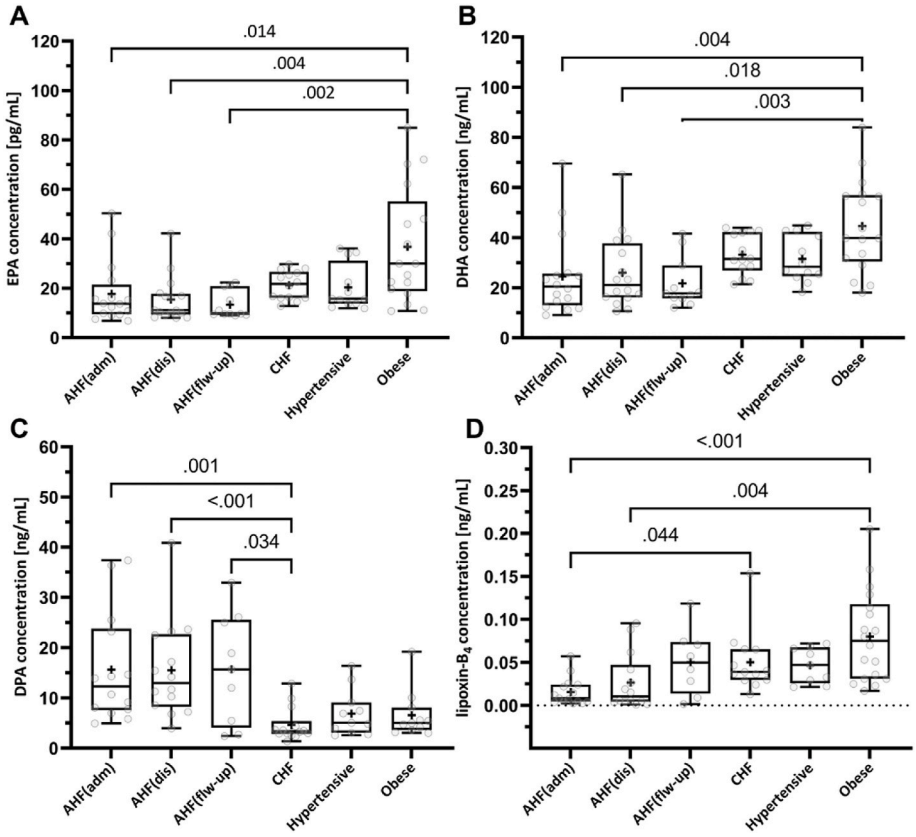

Fig. 2. Box plots of PUFAs A) EPA; B) DHA; C) DPA, and oxylipin D) lipoxin-B_4, which differed significantly among groups: AHF at admission (AHF(adm)), AHF at discharge (AHF(dis)), AHF at the follow-up visit (AHF(flw-up)), CHF, hypertensive, and obese; ($p < 0.05$, significantly different). The box-plot shows: the minimum, 25th percentile, median, 75th percentile, and maximum value for each variable investigated. The plus symbol inside the box shows the mean value.

Apart from being susceptible to ROS, mitochondria are emerging key players in the regulation of innate immunity. Studies on mitochondrial-mediated regulation of inflammation in CVDs are increasing [51]. Endogenous lipids are among the most important mediators involved in the regulation of all the phases of inflammation including initiation and resolution. Lipids are not just the major constituents of cell membranes and sources of energy, but are also key pathophysiological mediators of several intercellular and intracellular processes [52]. Among lipids, omega-3 PUFAs (e.g. ALA, EPA, DHA, n-3 DPA) have been associated with antiarrhythmic, antithrombotic, antiatherogenic and anti-inflammatory activities [53]. In mammals, the essential fatty acid ALA is enzymatically elongated and desaturated to produce EPA and subsequently DHA, with omega-3 DPA being the biosynthetic intermediate [54]. EPA and DHA exert an intense cardioprotective effect in a wide variety of CVDs, such as myocardial infarction and congestive heart failure. They enhance the production of NO and pro-resolving mediators (e.g. lipoxins, resolvins, protectins), inhibit ACE activity and thus, function as anti-inflammatory and anti-hypertensive molecules [55].

In our study obese patients were characterized by higher EPA levels than AHF. EPA has been found to exert a key role in the obese population by attenuating some symptoms of obesity through its antioxidant properties [53]. The caloric overload in obesity results not only in a notable lipid accumulation in adipose tissue as well as the circulation of free fatty acids, but also in an increased lipoprotein circulation [56]. Lipoproteins are associated with an endotoxin-scavenging role, thus

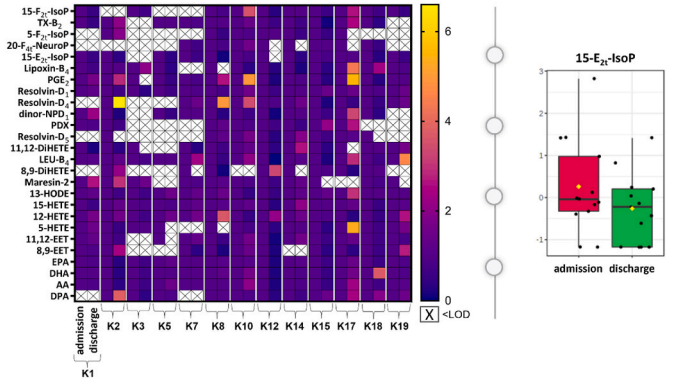

Fig. 3. Heatmap (left side) of the 26 features detected in saliva samples collected from thirteen AHF patients at both hospital admission and discharge (Kn). Data are normalized with respect to the admission point for each subject. Each square represents a collection point. Box plot (right side) of 15-E_{2t}-IsoP which differed significantly ($p < 0.05$) in AHF patients between admission and discharge. The box-plot shows: the minimum, 25th percentile, median, 75th percentile, and maximum value for each variable investigated. The yellow diamond inside the box shows the mean value. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

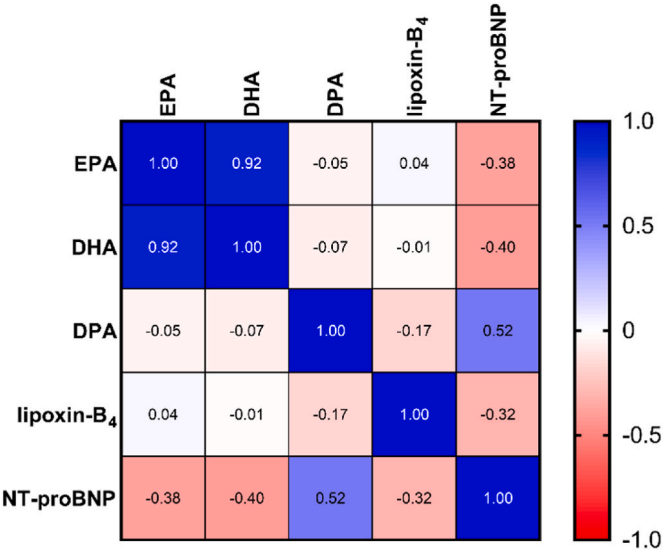

Fig. 4. Spearman correlation heatmap. Negative correlation (blank to red, r = 0 to −1.0) and positive correlation (blank to blue, r = 0 to 1.0) between the concentration level of the most significant oxylipins (EPA, DHA, n-3 DPA and lipoxin B_4) and the gold standard biomarker in heart failure blood NT-proBNP. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

preventing the harmful inflammatory consequences of endotoxemia. Traditional cardiovascular risk factors such as obesity and hypercholesterolemia are thus associated with greater survival among chronic HF patients. This is referred to as the "reverse epidemiology" paradox, which could be explained by the presence of the "malnutrition-inflammation complex syndrome" in chronic HF patients [57]. This suggests that nutritional strategies (i.e. omega-3 PUFAs diet supplementation) could be used in HF to maintain dietary adequacy, reduce the risk of hospitalization and improve survival, as clearly suggested by the European Society of Cardiology as well as the American College of Cardiology/American Heart Association [55].

Among omega-3 PUFAs, omega-3 DPA has gained increasing attention. The literature on both omega-3 DPA and its oxidation products (e.g., $diHDPA_{n-3}$) is limited because of the poor availability of the pure compound, nevertheless data suggests that omega-3 DPA does not only serve as a reservoir of DHA and EPA in humans [58], but it shows beneficial health effects [59]. In our study, n-3 DPA was found to be highly expressed in AHF subjects compared to CHF subjects. This was proven during hospitalization, i.e. from admission to discharge, but also at the follow-up sample collection point (after one month). Omega-3 DPA showed an opposite trend compared to EPA and DHA, which is consistent with its role as a biosynthetic intermediate product in the formation of DHA from EPA. Dietary habits, e.g. fish consumption, could impact on circulating omega-3 PUFA levels in patients, more than inflammation status or disease severity. To assess the relationship between the increase in omega-3 DPA levels and the worsening of the clinical situation due to the re-acutisation episode in HF subjects, we evaluated the correlation between the omega-3 DPA concentration and NT-proBNP which is the gold standard biomarker in heart failure. We found a medium positive correlation between the two variables (Spearman r, 0.52) as clearly shown in Fig. 4. These results demonstrate that the decrease in omega-3 DPA in the acute setting from admission to follow-up was not only diet-related, but may be also a consequence of a lipid rearrangement typical of the failing heart during acute decompensation. If confirmed, this could open up a new scenario in the use of omega-3 DPA as a potential predictive marker of re-acutisation episodes, thus providing opportunities for preventive interventions and reduced hospitalizations.

Apart from PUFAs, many molecules play a central role in regulating the inflammatory response by controlling its magnitude and duration, such as the PUFA-derived pro-resolving mediators (SPMs) [60]. Endogenous SPMs appear to control the host response and ease the resolution of inflammation [61]. Lipoxins are an important class of lipid mediators with inflammation-resolving properties. Lipoxins are endogenous mediators of resolution that are locally produced at sites of inflammation in the early phases by the sequential lipoxygenation of arachidonic acid during cell–cell interactions [48]. To date, two types of native lipoxins, lipoxin-A_4 and its positional isomer, lipoxin-B_4, have been identified [48] and recognized as vasoactive molecules as well as mediators in the regulation of leukocyte functions [62].

In the present work the lipoxin-B_4 concentration varied significantly between AHF patients at hospital admission and CHF patients. In the acute setting patients showed lower levels of lipoxin-B_4, which gradually increased during the hospitalization, at the follow-up visit reaching comparable levels to the CHF population. Plasma levels of lipoxin-A_4 have been found to be downregulated in chronic HF in all the NYHA classes [48], showing a more pronounced decrease in line with the increase in disease severity. Reina-Couto et al. observed inverse correlations between plasma lipoxin-A_4 and the established HF biomarkers (e.g. BNP, troponin I, and myoglobin), as well as the markers of pro-inflammatory/pro-oxidant injury, such as CRP and uric acid [63]. In our case, we found a medium negative correlation between lipoxin-B_4 and NT-proBNP (Fig. 4, Spearman r = −0.32). The reduction in lipoxin-B_4 concentration that we observed during the acute decompensation episode thus appeared to be reasonable, as well as its return to the levels of chronic subjects once stable clinical conditions were restored. These results suggest the counter-regulatory and protective role of lipoxins in HF pathophysiology, thus highlighting the importance of measuring these SPMs as tools for risk stratification [48]. During the healing phase, macrophages switch from the M1-phenotype to primarily anti-inflammatory macrophages, and then to reparative M2 macrophages, which are the main ones responsible for SPM production [54]. As critical regulators of the resolution program, targeting the actions of macrophages could help control inflammation and cardiac remodelling in the context of cardiac healing. We believe that the targeted modulation of endogenous mechanisms of inflammation, rather than its simple suppression, will soon become a key approach to treating chronic inflammatory diseases.

4. Conclusions

Our MS-based platform was shown to be suitable for the simultaneous quantitation of sixty salivary lipid mediators in CVDs samples. A lipid rearrangement typical of the failing heart during acute decompensation was clearly evidenced from our preliminary results. The targeted regulation of the inflammatory storm, rather than the simple suppression of the inflammation, could be the most successful approach for the management of CVDs in the near future.

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Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contribution

D.B., S.G., T.L., F.D.F.: Conceptualization; D.B., S.G., A.L.: Methodology; D.B., T.L., S.G., P.S.: Formal analysis and investigation; D.B.: Writing - original draft preparation; D.B., S.G., T.L., A.B., F.V.: Writing - review and editing; T.D., C.O., J-M.G., L.B.: Resources; T.L., F.D.F.: Supervision.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2023.03.015.

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