Ergebnisbericht gemäß § 42b AMG

- Synopse -

Version 01, Datum 30.09.2021

Velphoro and impact on the oral cavity and gut microbiome

Microbiom-Eisen

Multi-center, prospective, controlled, open, non-randomized, 2-arm-parallel, interventional, exploratory pilot study

EudraCT Nummer: 2017-003240-20

Vorlage-Nummer: 4042366

Kurztitel: Microbiom-Eisen

Sponsor der klinischen Prüfung:
RWTH Aachen, vertreten durch den Rektor, vertreten durch den Dekan der Medizinischen Fakultät Univ.- Prof. Dr. rer. nat. Stefan Uhlig

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Unterschriften

Die unterzeichnenden Autoren stimmen den Inhalten des vorliegenden Ergebnisberichtes durch ihre Unterschriften zu. Die hier berichtete, klinische Prüfung wurde nach den Grundsätzen der Deklaration von Helsinki, der Guten Klinischen Praxis (GCP) sowie den geltenden Gesetzen durchgeführt.

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Unterschrift _____ Ort, Datum _____

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Handelsname des Arzneimittels	Velphoro®
Wirkstoff/ Aktive Substanz	Sucroferric Oxyhydroxide
Titel der Studie	Velphoro and impact on the oral cavity and gut microbiome
Prüfer der klinischen Prüfung	Univ.- Prof. Dr. med. Jürgen Floege Department of Nephrology and Clinical Immunology University Hospital RWTH Aachen
Studienzentrum/-zentren:	University Hospital RWTH Aachen Department of Nephrology and Clinical Immunology Pauwelsstraße 30, 52074 Aachen
Publikationen	None (but a manuscript is in preparation)
Studienzeitraum	First-Patient-In: 27.02.2018 Last-Patient-Out: 30.12.2019 For each individual patient, study-related activities will be carried out for 4-5 weeks and for healthy subjects on only one day. The study has been paused in May 2020 due to the SARS-CoV-2 pandemia. Recruitment was restarted in July 2020, yet due to insufficient recruitment the study has been terminated early in October 2020.
Phase der klinischen Prüfung	IV
Art des Vorhabens	This pilot study will be carried out to determine if the regular intake of iron-based Velphoro® by hyperphosphatemia patients influences the microbiome in the oral cavity and/or the gut. After study inclusion, patients will undergo a dental investigation and two samples of blood, saliva, gingival biofilm (plaque) and stool will be collected, respectively.

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	Then, patients will be switched to the Velphoro® medication directly from their prior phosphate binder medication, i.e. no wash-out period will be included. Samples (as described above) will be collected after one and after four weeks following treatment initiation. An additional dental investigation will be carried out prior to the “4-week sample collection”. Afterwards, patients will return to their former medication carried out previously or may stay on Velphoro medication if preferred. Healthy subjects will only undergo a dental investigation after study inclusion and two samples of saliva, gingival biofilm (plaque) and stool will be collected, respectively. For evaluation, the pH value and $Fe^{2+/3+}$ contents are determined in the saliva samples. To investigate the distinct microbiome compositions, DNA will be isolated from collected samples (saliva, gingival plaque and stool) and used as template for a bacterial polymerase chain reaction (PCR). The V3-V4 region of ribosomal 16S DNA will be amplified and the fragments will be analyzed by Next Generation Sequencing (MiSeq, Illumina). The abundances and relative frequencies of distinct bacteria will be determined. Study Flow Chart (next page)

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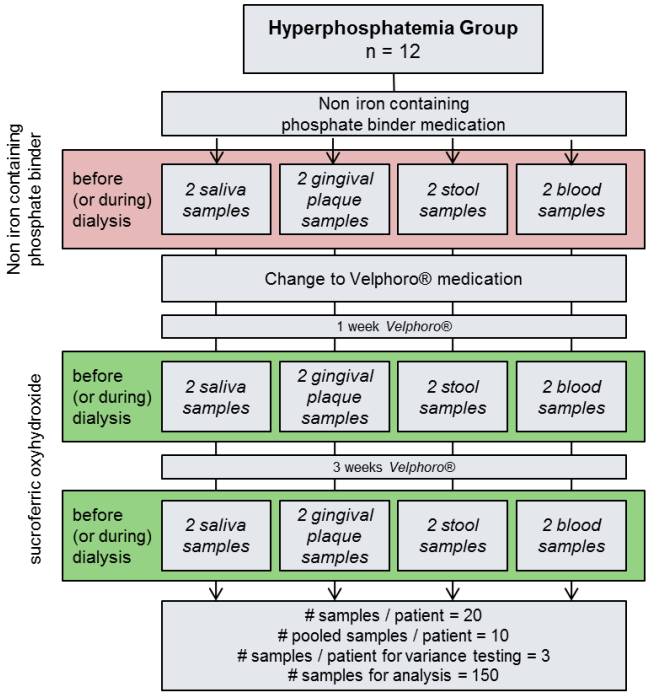

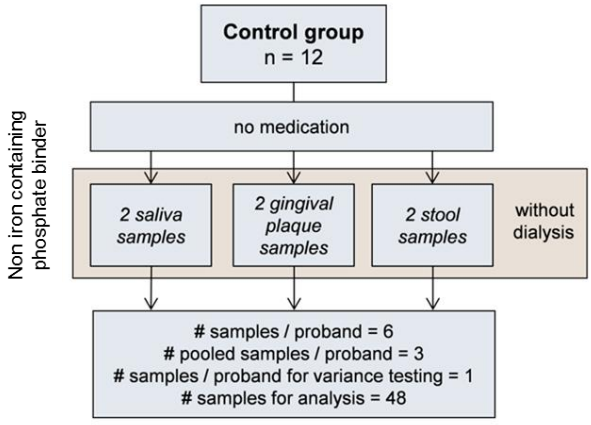

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	Visit Schedule:

Activity	**Visits**		
	V0 Screening/	**V1 Day 7**	**V2 Day 28**
In/exclusion criteria	X		
Written informed consent	X		
Demographics, Medical history	X		
Physical examination	X		
Vital signs	X	X	X
Concomitant medication	X		
Pregnancy test	X		
Dental investigation	X		X
Sampling	X	X	X
Chemical analysis	X	X	X
Dialysis	X	X	X
Blood withdrawal	X	X	X
Start of Velphoro® medication	X		
End of Velphoro® medication			X
Adverse Events		X	X

Studienziele	Primary objectives: Determination of abundances and relative frequencies of distinct bacteria in the collected

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	samples from patients and the control group. Based on these data, potential shifts in the microbiome during Velphoro® medication will be assessed. Secondary objectives: Determination of diversity measures describing the distribution of bacterial species within the microbiome (i. e. alpha-diversity, beta-diversity, Simpson index, Shannon-Wiener index), as well as the presence or absence of other medically relevant bacterial species (e.g. Pseudomonas, Enterobacteriaceae, Campylobacter etc.).
Primärer Zielparameter	Determination of abundances and relative frequencies of distinct bacteria in the collected samples from patients and the control group. Based on these data, potential shifts in the microbiome during Velphoro® medication will be assessed.
Sekundäre Zielparameter	Determination of diversity measures describing the distribution of bacterial species within the microbiome (i. e. alpha-diversity, beta-diversity, Simpson index, Shannon-Wiener index), as well as the presence or absence of other medically relevant bacterial species (e.g. Pseudomonas, Enterobacteriaceae, Campylobacter etc.).
Studiendesign	This is a multi-center, prospective, controlled, open, non-randomized, 2 arm parallel, interventional, exploratory pilot study.
Prüfmedikation / Behandlungsstrategie	Name of finished product: Velphoro® Name of active substance: Ferrum (sucroferric oxyhydroxide)

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Handelsname des Arzneimittels	Velphoro®
Wirkstoff/ Aktive Substanz	Sucroferric Oxyhydroxide
	Dose: 500 mg 3x daily as chewable tablet Indication: Hyperphosphatemia Mode of administration: oral Batch number(s): 6422011YA, 7410011BA
Behandlungsdauer	For each individual patient, study-related activities will be carried out for 4-5 weeks and for healthy subjects on only one day.
Vergleichsbedingung/ -medikation	none Name of finished product: n.a. Name of active substance: n.a. Dose: n.a. Indication: n.a. Mode of administration: n.a. Batch number(s): n.a.
Gesamtzahl Patienten	planned: 24 screened: 22 enrolled: 16 Drop-outs: 7
Studienpopulation	The study is planned in a case-control design with two groups: 1) hemodialysis patients suffering from hyperphosphatemia (n = 10) and 2) a control group of 10 age- and sex- and oral disease status-matched (dental caries and periodontal disease)

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	subjects with normal renal function. They were planned to be recruited during the clinical routine in the Department of Operative Dentistry, Periodontology and Preventive Dentistry.
Einschlusskriterien	Subjects, fulfilling the following inclusion criteria are suitable for participation in the study: <u>Patients:</u> • Suffering from hyperphosphatemia • Current treatment with a stable dose of a non-iron containing phosphate binder, e.g. Calcium acetate®, Calcium carbonate, Phosphonorm®, Fosrenol®, Renagel®, Renvela® • No or only parenteral iron application • Age of ≥ 18 years • Written informed consent prior to study participation • The subject is willing and able to follow the procedures outlined in the protocol <u>Control group:</u> • Normal renal function • No hyperphosphatemia • Age- and sex-matched and oral disease status-matched (dental caries and periodontal disease) in comparison to the hyperphosphatemia group • Written informed consent prior to study participation

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	• The subject is willing and able to follow the procedures outlined in the protocol
Ausschlusskriterien	Subjects, fulfilling one or more of the following exclusion criteria will not be included in the study: • Age less than 18 years • Currently on **oral** iron application • Antibiotic treatment within the last two months • Severe medical events within the last three months • Planned surgery for the duration of the sampling • Acute/chronic gastrointestinal infections • Smokers • Oral candidiasis • Oral cancer • Pregnant and lactating females • Haemochromatosis history • Committed to an institution by legal or regulatory order • Participation in a parallel interventional clinical trial • Receipt of an investigational drug within 30 days prior to inclusion into this study • The subject is mentally or legally incapacitated <u>Only for the patient group:</u> • Allergy to Velphoro® • Never got any phosphate binder

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	• Celiac disease or any other chronic inflammatory bowel disease • Previous major surgery in the gastrointestinal tract
<u>Kriterien zur Bewertung der</u> Sicherheit	Safety assessments will consist of monitoring and recording all adverse events and serious adverse events, regular measurement of vital signs and the performance of physical examinations. The study can be prematurely terminated if one of the following aspects is applicable: • Non- adherence to the study protocol, the declaration of Helsinki, ICH-GCP and/or applicable regulatory requirements • Missing compliance, patient does not follow instructions by the study team • Withdrawal of informed consent • Safety and well-being of the patients is not ensured any longer • Subject enrolment is unsatisfactory • The risks and benefits of continuing the study have been reassessed, and the risk outweigh any potential benefits • The incidence of AEs constitutes a potential health hazard to the subjects • Inclusion and exclusion criteria are not applicable any more

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	• Other reasons that prohibit a further participation according to an investigator's assessment Reason, time point, and specific reason for premature study termination of each subject will be documented. The investigator should determine a primary reason for premature study termination of each subject. All relevant safety data until subject's study termination will be collected and reported. The study will entirely be terminated in case the risk-benefit-ratio changes in such way, that premature study termination is indicated in order to protect subject's health.
Kriterien zur Bewertung der Wirksamkeit	The investigation of the efficacy of Velphoro® treatment is not a part of this study and has been investigated in former studies; Velphoro® is administered within the label of this drug, i.e. dialysis patients with hyperphosphatemia. For objectives and endpoints of this study see above.
Statistische Methoden:	This is a pilot, hypothesis generating trial. Therefore, a power analysis cannot be performed due to missing data in humans for the proposed endpoint. For a statistical analysis of generated data, percentage frequency data can be incorporated in any number of statistical packages, such as the R project, MeV, SAS, and QIIME.
Wesentliche Prüfplanänderungen:	• Amendment approved 06/2018: additional blood sampling during dialysis

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	• Amendment approved 10/2018: extension of recruitment time • Amendment approved 05/2019: additional study center • Amendment approved 06/2019: extension of study duration • Amendment approved 04/2020: harmonization of study documents due to optional blood sampling • Amendment approved 05/2020: recruitment stop • Amendment approved 07/2020: restart of recruitment • Official decree 08/2020: recruitment stop of undefined length following a review of the CTC-Aachen by Bezirksregierung Köln

SUMMARY:

Efficacy results:

Because of several recruitment stops the recruitment of healthy controls was unsuccessful, therefore **no samples of the control group were taken and analyzed and no control-results can thus be reported**.

Nevertheless, by comparing the results of one (t1= time, V1= sample) and four weeks (t2= time, V2= sample) on sucroferric oxyhydroxide (Velphoro®, hence abbreviated SFO) with baseline (t0= time, V0= sample) we were still able to answer relevant questions regarding primary and secondary outcome parameters.

Clinical characteristics of the study population

Of initially 16 enrolled patients, 11 patients were included in the final analysis. In Table 1 the patients included or excluded into analysis and justification for in- / exclusion from analysis are listed.

Table 1. Listing of inclusion or exclusion form analysis and justification for each patient.

Patient-ID	**Inclusion into / exclusion from analysis**	**Justification**
A-01	Inclusion	Completed study per protocol
A-02	Inclusion	Completed study per protocol
A-03	Exclusion	Drop-out after t0 due to adverse event, insufficient data for analysis
A-04	Exclusion	Drop-out after t0 due to adverse event, insufficient data for analysis
A-05	Inclusion	Completed study per protocol
A-07	Exclusion	Drop-out after t0 due to withdrawn consent, insufficient data for analysis
A-08	Inclusion	Completed study per protocol
A-09	Inclusion	Completed study per protocol

A-12	Inclusion	Completed study per protocol
A-13	Inclusion	Completed study per protocol
A-14	Exclusion	Drop-out after t0 due to withdrawn consent, insufficient data for analysis
A-15	Inclusion	Completed study per protocol
A-16	Inclusion	Drop-out after t1 due to adverse event, sufficient to answer relevant questions, although t2 data are missing
A-17	Inclusion	Drop-out after t1 due to serious adverse event (death, unrelated to study medication), sufficient data to answer relevant questions, although t2 data are missing
A-18	Exclusion	Completed study per protocol, but insufficient data for analysis due to missing samples (no stool production)
A-19	Inclusion	Drop-out after t1 due to adverse event, sufficient data to answer relevant questions, although t2 data are missing

Thus, 11 patients were included in the final analysis. At the analyzed patients' baseline (t0=0) the mean age was 66 years, one of the patients had diabetes mellitus, mean serum phosphate was 1.88 (mmol/L), mean hemoglobin 10.82 (g/dl), mean serum Fe 210 (µg/dl), mean transferrin saturation 22.49 (in%), mean serum PTH (n=10) 287,68 (pg/ml), and mean ferritin 559,66 (ng/ml). For further details please see **Table 3.** Baseline characteristics of all enrolled patients can be found in **Table 2**. Dental investigation was carried out at baseline and prior to t2-sampling (week 4). Here, all parameters were examined again. In particular, attention was paid to whether changes such as discoloration/staining were present after taking SFO. A comparison of both oral investigations revealed that the number of DMFT (caries index) and PSI (periodontal index) scores did not change after taking SFO. There was no pigmentation or staining of the gingiva. Mild and reversible tooth staining was found in some of the patients. Only one patient had lost a tooth between two visits, which was extracted by the dentist because of reasons not related to SFO intake.

Table 2. Clinical baseline characteristics of all patients.

Patient	Age	Duration	DM/non DM	DW (Kg)	Protein (g/dl)	P (mmol/l)	iCa (mg/dl)
A01	44	12-24 months	non-DM	80.5	6	2.1	1.11
A02	58	12-24 months	DM	115,5	7.2	1.77	1.06
A03	51	>36 months	non-DM	39,8	6.7	1.94	1.31
A04	79	>36 months	non-DM	86	5.9	1.44	1.25
A05	83	>36 months	non-DM	67	6.4	1.7	1.36
A07	48	>36 months	DM	74	6.5	1.67	1.09
A08	79	12-24 months	non-DM	76.5	5.8	1.15	1.13
A09	43	12-24 months	non-DM	81	n.d.	2.87	n.d.
A12	64	24-36 months	non-DM	71.5	5.9	2.9	1.11
A13	77	36-48 months	non-DM	86	n.d.	1.58	n.d.
A14	36	>48 months	non-DM	102	n.d.	3.62	1.09
A15	66	>48 months	non-DM	116	7.1	1.33	1.11
A16	56	36-48 months	non-DM	77	n.d.	2.08	n.d.
A17	80	36-48 months	non-DM	60	6.3	1.39	0.99
A18	61	>48 months	non-DM	88	5.8	2.08	1.16
A19	76	>48 months	non-DM	59	5.9	1.77	1.04

	Ca (mmol/L)	PTH (pg/ml)	Hb (g/dl)	Fe (ug/dl)	TSAT (%)	Ferritin (ng/ml)	pH	HCO3 (mmol/l)
A01	2.01	278	10.8	58	18.6	57.6	7.34	22.8
A02	2.11	398.8	9.7	34.4	9.5	117.7	7.43	27.9
A03	2.42	56	10	127	53.6	256.2	7.299	24.4
A04	2.28	935.8	10.5	45.6	15.7	66	7.414	23.2
A05	2.68	265.7	9.6	33.2	13.2	606.2	7.42	27.6
A07	1.96	n.d.	11.2	94.4	30.3	144.5	7.418	27.6
A08	2.19	138.2	11.9	74.2	32.5	996.8	7.42	23.9
A09	2.49	384	10.8	505	22	753	n.d.	n.d.
A12	2.19	188.9	13.3	78.9	20	80	7.35	19.7
A13	2.25	n.d.	11.6	662	24	1171	n.d.	n.d.
A14	2.4	n.d.	11.2	366	12	126	7.281	18.5
A15	2.32	150.8	10.4	98.5	31	584.1	7.35	19.7
A16	2.24	649	11.1	668	36	954	n.d.	n.d.
A17	1.94	194.9	9.0	33	14.5	481.4	7.41	20.8
A18	2.34	138.8	9.6	44.3	15.3	444.5	7.415	26.3
A19	1.98	228.5	10.8	68	26.1	354.5	7.32	23.9

Legend:

DM = diabetes mellitus, DW = dry weight on dialysis, Alb = albumin, P = phosphate, iCa = ionized calcium, Ca = total calcium, Hb = hemoglobin, Fe = iron, TSAT = transferrin saturation, HCO3 = bicarbonate

Table 3. Clinical baseline characteristics of analyzed patients.

Patient	Age	Duration	DM/nonDM	DW (Kg)	Protein (g/dl)	P (mmol/l)	iCa (mg/dl)
A01	44	12-24 months	non-DM	80.5	6	2.1	1.11
A02	58	12-24 months	DM	115,5	7.2	1.77	1.06
A05	83	>36 months	non-DM	67	6.4	1.7	1.36
A08	79	12-24 months	non-DM	76.5	5.8	1.15	1.13
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A13	77	36-48 months	non-DM	86	n.d.	1.58	n.d.
A15	66	>48 months	non-DM	116	7.1	1.33	1.11
A16	56	36-48 months	non-DM	77	n.d.	2.08	n.d.
A17	80	36-48 months	non-DM	60	6.3	1.39	0.99
A19	76	>48 months	non-DM	59	5.9	1.77	1.04

	Ca (mmol/L)	PTH (pg/ml)	Hb (g/dl)	Fe (ug/dl)	TSAT (%)	Ferritin (ng/ml)	pH	HCO3 (mmol/l)
A01	2.01	278	10.8	58	18.6	57.6	7.34	22.8
A02	2.11	398.8	9.7	34.4	9.5	117.7	7.43	27.9
A05	2.68	265.7	9.6	33.2	13.2	606.2	7.42	27.6
A08	2.19	138.2	11.9	74.2	32.5	996.8	7.42	23.9
A09	2.49	384	10.8	505	22	753	n.d.	n.d.
A12	2.19	188.9	13.3	78.9	20	80	7.35	19.7
A13	2.25	n.d.	11.6	662	24	1171	n.d.	n.d.
A15	2.32	150.8	10.4	98.5	31	584.1	7.35	19.7
A16	2.24	649	11.1	668	36	954	n.d.	n.d.
A17	1.94	194.9	9.0	33	14.5	481.4	7.41	20.8
A19	1.98	228.5	10.8	68	26.1	354.5	7.32	23.9

Legend:

DM = diabetes mellitus, DW = dry weight on dialysis, Alb = albumin, P = phosphate, iCa = ionized calcium, Ca = total calcium, Hb = hemoglobin, Fe = iron, TSAT = transferrin saturation, HCO3 = bicarbonate

Microbiome results:

The 0-hypothesis of our study was that no changes in the microbiome (oral, intestinal) occur during Velphoro-administration. The alternative hypothesis was that bacterial taxa usually living under Fe- deficit will profit and those without deficit (e.g. producers of siderophores and/or metal-sampling alternatives) will relapse after SFO and thus Fe(III) uptake.

RESULTS primary objectives

To re-state, the primary objectives were: *Determination of abundances and relative frequencies of distinct bacteria in the collected samples from patients [and the control group, excluded, see above]. Based on these data, potential shifts in the microbiome during Velphoro® medication will be assessed.*

Overall, the microbiome is not significantly altered with patient-, specimen-, and microbial-taxon specific exceptions

We obtained 30 saliva, 30 biofilm, and 30 fecal samples (with 11 samples/patient at t0 and t1 and 8 samples at t2, thus three drop-outs without withdrawing formal consent, of which one patient unfortunately died because of reasons not related to the study) and an average of 24,650 (saliva), 24,356 (biofilm), and 19,538 (feces) sequence reads per sample. This is about four times the content of the only comparable study so far (Iguchi et al. 2020), a study focusing on the intestinal microbiome of SFO-patients only and the sequence information generated here can thus be regarded as success. Clustering, and non-metric multidimensional scaling (MDS) analysis of β-diversity revealed that most differences between microbiomes were patient- and/or specimen-dependent (for details see Figure 1).

Summarizing the main overall results of primary outcomes-analysis: relevant shifts (**in total 12 events**) in microbiome were seen in patients A01 (saliva, biofilm), A02 (biofilm, feces), A09 (biofilm), A12 (saliva, biofilm, feces), A13 (saliva, biofilm), and A16 (biofilm, feces, with data for t0/1 only). Henceforward, we name these patients *shifters*. In contrast, the microbiomes of patients A05, A08, A15, A17, and A19 (labelled *non-shifters*) appeared quite stable after SFO administration and independent of the kind of specimen investigated. However, in saliva of A15 and feces of A08, the microbiome changed from t0 to t1, but the t2-microbiome clustered again with t0 (boomerang move). **In response to SFO-uptake, a patient classification into shifters and non-shifters is a central result of this pilot study.**

RESULTS secondary objectives (short, primary version)

The secondary objectives were: *Determination of diversity measures describing the distribution of bacterial species within the microbiome (i. e. alpha-diversity, beta-diversity, Simpson index, Shannon-Wiener index), as well as the presence or absence of other medically relevant bacterial species (e.g. Pseudomonas, Enterobacteriaceae, Campylobacter etc.).*

In-depth analysis of changes in the oral microbiome after SFO administration

Overall, sequencing revealed no obligate pathogens or medically relevant bacterial species in relevant numbers. Even *Escherichia* (Escherichia-Shigella) was only sporadically found in feces (t0: mean 0.1%, median 0.0%; t1: 0.2%, 0.0%; t2: 0.0%, 0.0%).

Sequencing revealed 8 different major phyla **in the saliva samples**, dominated by Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria as well as Campylobacterota (non-or low-pathogenic Campylobacter), Candidatus Patescibacteria, and Cyanobacteria ordered by abundance from high to low. We also identified 15 major genera, namely Streptococcus, Rothia, Veillonella, Actinomyces, Neisseria, Prevotella, Granulicatella, Haemophilus, Leptotrichia, Gemella, Fusobacterium, Atobobium, Porphyromonas, Alloprevotella, and Campylobacter.

The **biofilm** showed a greater richness and 12 different major phyla. Here, the microbiome was dominated by Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Proteobacteria as well as Campylobacterota, Candidatus Patescibacteria, Spirochaetes, Synergistetes, Desulfobacterota, Chlorobacteria, and Euryarchaeota (the latter from the domain of archaea), again ordered by abundance. We again identified 15 major genera: Actinomyces, Streptococcus, Veillonella, Prevotella, Rothia, Leptotrichia, Selenomonas, Candidatus F0332, Corynebacterium, Neisseria, Fusobacterium, Capnocytophaga, Porphyromonas, Bifidobacterium, and oral, low-pathogenic Campylobacter.

The **fecal samples** showed an intermediate richness and 10 different major phyla. Here, the microbiome was dominated by Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Proteobacteria as well as Desulfobacterota, Cyanobacteria, Euryarchaeota, Fusobacteria, and Synergistetes. The most abundant 15 genera were an unknown Lachnospiraceae, Blautia, Faecalibacterium, Ruminococcus, Bifidobacterium, Subdoligranulum, Christensenellaceae R-7 group, Alistipes, Akkermansia, Neisseria, Lachnospiraceae NK4A136 group, genus UCG-002, Anaerostipes, Ruminococcus torques group, and Collinsella.

As outlined above, changes in the microbiome were mainly patient (shifters, non-shifters) and specimen-specific (with the most dynamic seen within biofilm, followed by fecal samples, and saliva). We therefore analyzed the **most significant microbiome-changes in certain specimens of *shifters* in more depth and here are the results.**

As expected, the overall changes in microbiome were driven by changes in a few genera, obviously reacting on the Fe-plethora at t1, but also seen in saliva to some extent. Significant changes over all bacterial taxa (on species, genus, and family level) and over all patients are summarized and visualized in **Figures 1-3** and significances calculated in **Figure 4** at the end of this report. The concentration of Fe(2+) and Fe(3+) - as the most likely driving force for these changes- is visualized in **Figure 5:**

The eight significant unidirectional (t0 to t1 to t2) changes observed in this study were increase of **Streptococcus salivarius** (p<.0439), and **oral Prevotella** (p<.0302) in saliva, decrease of **Corynebacterium** (p<.0498), **Capnocytophaga** and **Neisseriaceae** (latter both p<.0313) in biofilm, and increase of **intestinal Veillonella** and **Ruminococcus torques group** (both p<.0351) as well as decrease of **Subdoligranulum** (<.0496) in fecal samples. **Therefore, the Fe-dependent metabolism of these eight bacterial taxa will be discussed in depth**. Furthermore, and not further discussed here: Atopobium parvulum in saliva, Veillonella in biofilm, and Eubacterium coprostanoligenes group as well as Blautia sp. clone B2-11, even while showing significant changes, were excluded from further analysis as their dynamic was reversible (increasing to t1 and decreasing to t2 or vice versa) and not unidirectional and thus very difficult to interpret.

Main secondary outcome of study: taxa which significantly profited from iron-plethora but do not possess siderophores: S. salivarius and obligate anaerobes Prevotella, Veillonella, Ruminococcus torgues

From many streptococci it is known that they do not synthesize known siderophores but are able to use ferric ferrichrome, a hydroxamate siderophore produced by other bacteria in the niche, as an Fe source [1]. For instance, several iron transport systems have been predicted in S. pneumoniae by comparative genomics but siderophores have not been identified [2]. In a Pseudomonas (a prominent siderophore producer) and Streptococcus spp. co-culture cystic-fibrosis model it was demonstrated that streptococci can easily be outcompeted by overproduction of siderophores from competitors limiting iron [3]. Anaerobic genera do not produce siderophores and usually profit from siderophore supplementation. For example, addition of various siderophores to culture media was found to increase the growth of

fastidious organism and especially of oral Prevotella (HOT-376) [4]. In the cases of anaerobic Veillonella and Ruminococcus torques we performed an extensive, advanced PubMed search revealing no evidence of siderophore production. On the molecular level, mining the GenBank annotations of Veillonella atypica gut isolate MGYG-HGUT-01444 (accession number: CABKSO000000000) or Ruminococcus torques strain AM22-16 (accession number: NZ_QRIH01000001.1) reference genomes revealed again no siderophores. In a recent study investigating the impact of ferric citrate, as an alternative to calcium carbonate phosphate binders in eight patients, it was shown that members of Flavonifractor, Cronobacter but also certain Ruminococcus sp. were enriched in patients treated with ferric citrate phosphate binder [5]. Taken together, it is plausible that out-competition of siderophore-non-producers such as S. salivarius or anaerobes is reduced under iron plethora such as in our study and the 0-hypothesis is supported. Comparable to our study, Iguchi et al. found no overall significant change in the intestinal bacterial diversity. However, they found a significant increase of families Clostridiaceae (0.6%, 2.6%, P = 0.0264) and Oscillospiraceae (0.21%, 0.40%, P = 0.023) and of genus Oscillibactor (t0 0.20%, t12weeks 0.44%, P = 0.022) [6]. Interestingly, at least the abundance and increasing dynamic of Oscillibacter was similar in our study (t0 0.22%, t1week 0.30%, t4weeks 0.42%). After screening literature and a reference genome O. valericigenes strain Sjm18-20, it can be concluded that this strictly anaerobic genus does not produce any metal binding chelators supporting our model.

Main secondary outcome of study: taxa which significantly relapsed under iron-plethora and do possess siderophores or alternatives: Corynebacterium matruchotii, Capnocytophaga, Neissseriaceae, Bifidobacterium and Subdoligranulum

On the other hand, Corynebacterium matruchotii (reference genome ATCC14266), the by far most dominating species in the oral biofilm [7], produces siderophore biosynthesis proteins of the IucA/IucC family and corresponding Fe(III)-siderophore ABC transporter permeases (genes HMPREF0299_RS00335 and HMPREF0299_RS01575). Under Fe(III) plethora the energy loss by continued production of such needless proteins is disadvantageous and the penalty paid is revealed by our study.

However, all other three decreasing taxa do not produce siderophores but instead are known for alternative Fe(III) capture systems: Capnocytophaga possess a polysaccharide utilization locus (PUL) which can act as an alternative iron capture system (ICS) [8]. Neisseria

(pathogenic species N. meningititis and N. gonorrheae studied in depth) – even aerobic - does not produce siderophores. However, Neisseria has developed alternatives to siderophores. The ferric binding protein (FbpA) is one of the major proteins regulated by the level of environmental iron in the genus Neisseria. Its conservation in all species of pathogenic Neisseria has been demonstrated [9]. We searched apathogenic, commensal Neisseria spp. and detected FbpA in N. lactamica (strain HMT-649; gene accession no. NZ_CP031253.1 position: 400,598). Next, the correspondent FASTA gene sequence was used to blast against Neisseriaceae (taxid 481) and - besides the three species mentioned above - the commensal oral N. mucosa (HMT-682) and N. cineria (HMT-956) were found to possess FbpA as well. As outlined above, there is evidence that FbpA (or other so called periplasmatic binding proteins, PBP) reaches the bacterial surface and helps sampling Fe(III) directly from the environment [10]. Finally, the iron dependency of Subdoligranulum was studied. The taxon, represented by reference species S. variabile DSM15176, was described in 2004 [11]. The nearest named relative corresponded to Faecalibacterium prausnitzii. Even though staining gram-negatively, this organism is phylogenetically a member of the gram-positive Clostridium leptum supra-generic rRNA cluster. Since its description and inclusion into microbiome-databases and popular bioinformatics pipelines it was frequently reported as a semi-abundant member of the human fecal flora [12]. By blasting the genome of reference strain DSM15176 we were able to identify a TroA-like metal receptor (GenBank EFB75720.1) functioning as ABC transport of ferric siderophores but also for uptake of metal ions including Fe(III), which could at least partially explain the decrease in abundance in case of iron-plethora. Furthermore, Bifidobacterium showed a trend to reduction: i) saliva: at t0 mean 0.4%, median 0.2%; t1: 0.5%, 0.2%; t2: 0.3%, 0.1%, ii) biofilm at t0: 1.6%, 0.1%; t1: 2.0%, 0.1%; t2: 0.7%, 0.2%, iii) feces at t0 5,3%, 1.4%; t1: 2.5%, 0.9%; t2: 3.7%, 0.8%). Vazquez-Gutierrez et al. screened six different Bifidobacterium species for their siderophore activity which ranged from 3 to 89% siderophore units, with 35 strains (41%) exhibiting high, 31 (36%) intermediate and 20 (23%) low activity 26 [13]. Other potent siderophore producers such as Acinetobacter, Bordetella, Burkholderia, Mycobacterium, Pseudomonas, Serratia, Streptomyces, and Yersinia were not found in any sample. Escherichia (g__Escherichia-Shigella) was only sporadically found in feces (t0: 0.1%, 0.0%; t1: 0.2%, 0.0%; t2: 0.0%, 0.0%).

Safety results:

Listing of all AEs/SAEs:

Patient ID	Event	Start	End	Serious	Intensity	Causality	Action	Outcome
A-03	Diarrhea	25.05.2018	31.05.2018	2	2	2	4	1
A-03	Gastrospasm	25.05.2018	31.05.2018	2	2	2	4	1
A-04	Diarrhea	26.05.2018	29.05.2018	2	2	2	4	1
A-04	Gastrospasm	26.05.2018	29.05.2018	2	2	2	4	1
A-16	Vomiting	07.10.2019	08.10.2019	2	2	2	4	2
A-16	Nausea	07.10.2019	08.10.2019	2	2	2	4	2
A-17	Death	15.11.2019	15.11.2019	1	3	5	1	6
A-19	Diarrhea	23.12.2019	30.12.2019	2	2	2	4	1
A-19	Vomiting	23.12.2019	28.12.2019	2	2	2	4	1

Legend:

Serious: 1 = yes, 2 = no;

Intensity: 1 = mild, 2 = moderate, 3 = severe;

Causality: 1 = certain, 2 = probable / likely, 3 = possible, 4 = unlikely, 5 = no causal relation;

Action: 1 = none, 2 = dose reduction, 3 = interruption of medication and re-administration, 4 = preliminary termination, 5 = concomitant therapy, 6 = hospitalization;

Outcome: 1 = recovered / resolved, 2 = in recovery / improved, 3 = no change / ongoing, 4 = recovered with sequelae, 5 = unknown, 6 = death

Number of subjects affected and rate of occurrence of AEs/SAEs in each treatment group:

Event	Study Arm A: Velphoro® group	Study Arm B: Control group*
	Absolute number of subjects [rate of occurrence]	Absolute number of subjects [rate of occurrence]

All AEs	**4 [21.1 %]**	**n.a.**
Diarrhea	3 [15.8 %]	**n.a.**
Gastrospasm	2 [10.5 %]	**n.a.**
Vomiting	2 [10.5 %]	**n.a.**
Nausea	1 [5.3 %]	**n.a.**
All SAEs	**1 [5.3 %]**	**n.a.**
Death	1 [5.3 %]	**n.a.**

*due to several recruitment stops no healthy controls were enrolled in the study; n.a. – not applicable

CONCLUSION: In conclusion of the microbiological results, the 0-hypothesis of our study (no significant changes between baseline and t1/2) can be confirmed. Over all patients tested, changes in the microbiome after administration of sucroferric oxyhydroxide (Velphoro®) were not significant, and if present were transient and reversible. However, shifts described in our alternative hypothesis did occur, but were restricted to a few patients (6 out of 11 were *shifters*) and were not apparent in all - but usually in more than one - specimens tested. From the dental point of view a comparison of both oral investigations (t0 versus t2) revealed that neither the number of DMFTs (caries index) nor the PSI scores (periodontal index) increased. In addition, no more than minor staining was recognized after taking SFO.

Limitations of our study: There are several limitations in this study. First, the number of patients recruited in this pilot study and the heterogeneity by age, gender and oral conditions make every analysis rather difficult. The exclusion of a healthy match group was not essential but still hinders interpretation of microbiome data. Significant changes in the microbiome were found concentrated in four to five taxa producing siderophores or alternatives and four to five taxa with not such ability. This made us focus on siderophores here. However, the dynamic of iron within our body and in contact with the microbiome is extremely complicated and a single explanation can hardly be precise. In this context, it is important to consider that all microbial features are expressed at the strain level whereas the microbiome is monitored on much higher, usually the phylum, family or at least genus level.

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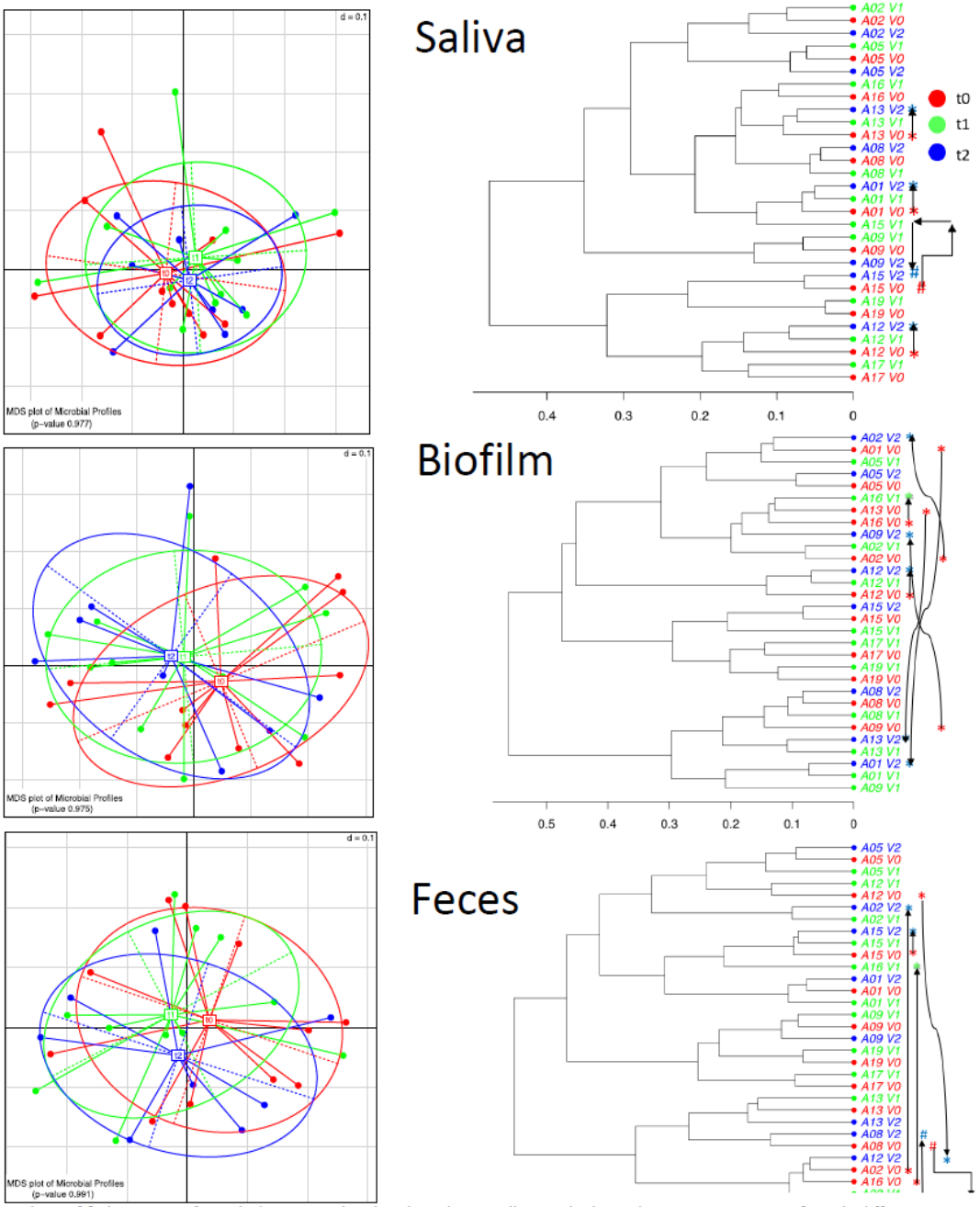

Figure 1. Analysis of β-diversity. **Left**: multidimensional scaling (MDS): over all taxa, the beta-diversity was not significantly different between the saliva, biofilm, and fecal samples collected at three different time points, t0 (before switch to SFO), t1 (one week after SFO switch), and t2 (four weeks after SFO switch). **Right**: Cluster analysis and intra-patient dynamical change of microbiome. Only significant changes are followed by arrows. Patients with such a shifting microbiome (named *shifters*) were A01 (saliva, biofilm), A02 (biofilm, feces), A09 (biofilm), A12 (saliva, biofilm, feces), A13 (saliva, biofilm), and A16 (biofilm, feces, with data for t0/1 only). Microbiomes of patients A05, A08, A15, A17, A19 (*non-shifters*) appeared quite stable, independent of specimen. In a subgroup here, however, two specimens (saliva of A15 and feces of A08) changed from t0 to t1, but t2-microbiome clustered again with t0, following a boomerang move.

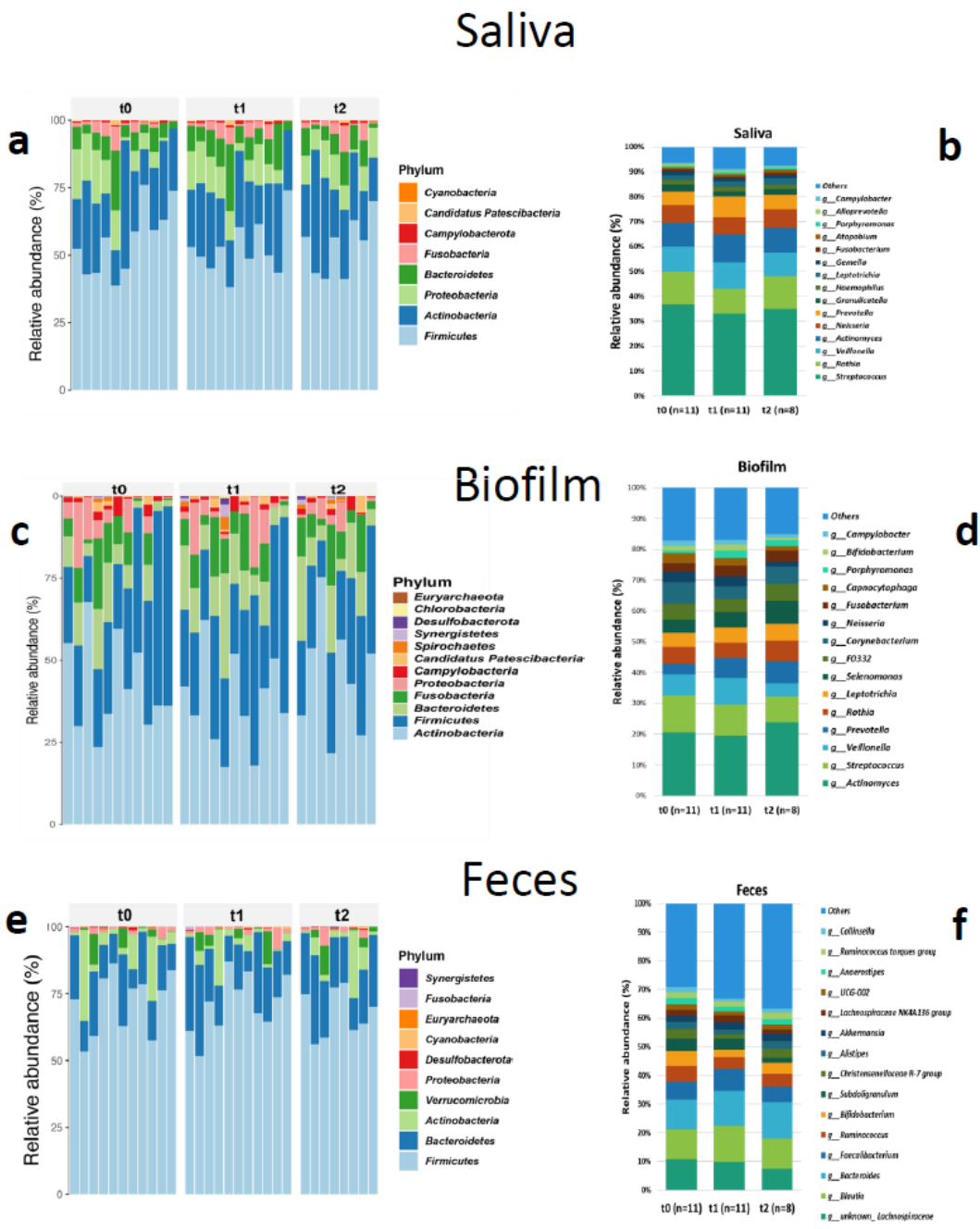

Figure 2. Changes in the oral (a-b saliva and c-d teeth-attached biofilm) and intestinal e-f microbiome from baseline (t0) to one (t1) and four (t2) weeks of SFO administration. Phylum composition is presented on the left (a, c, e) and genera composition on the right side (b, d, f).

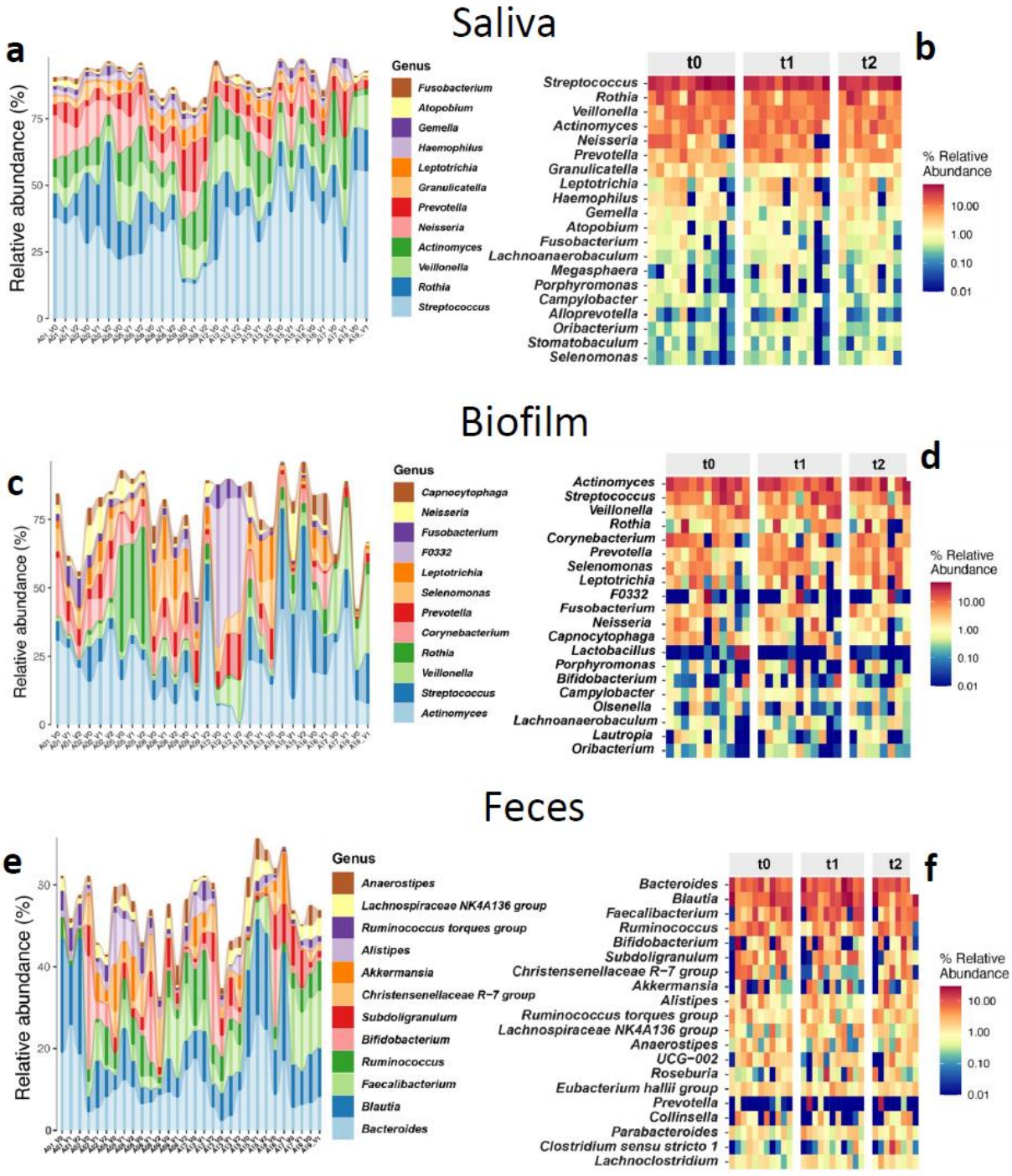

Figure 3. Changes in the oral (a-b saliva and c-d teeth-attached biofilm) and intestinal e-f microbiome from baseline (V0, t0) to one (V1, t1) and four (V2, t2) weeks of SFO administration. The patient-specific dynamic is presented on the left (a, c, e) whereas the relative abundances of each genus is presented on the right side (b, d, f).

3

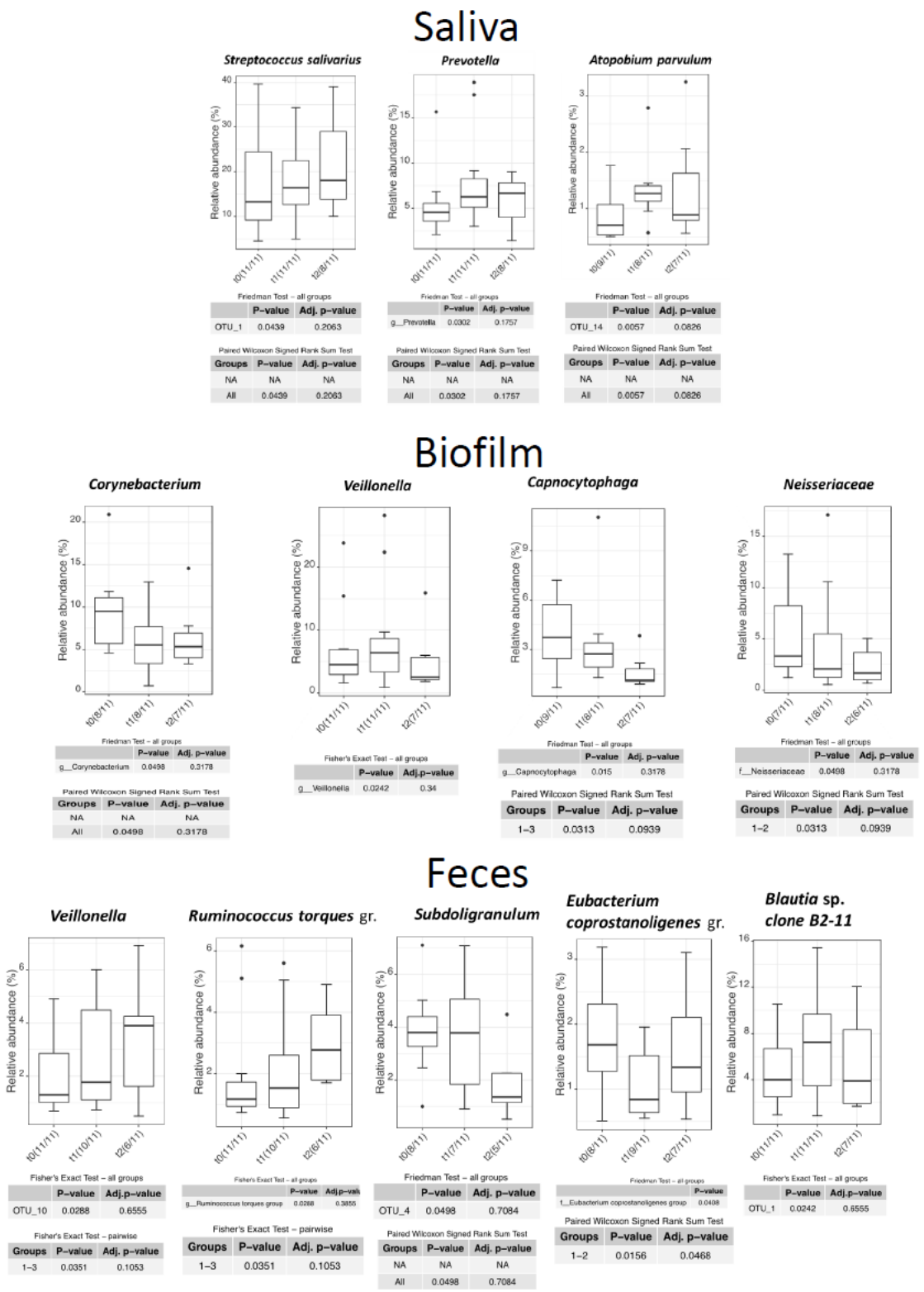

Figure 4. Statistically significant changes in certain taxa of the microbiome in saliva, biofilm, and fecal samples after SFO.

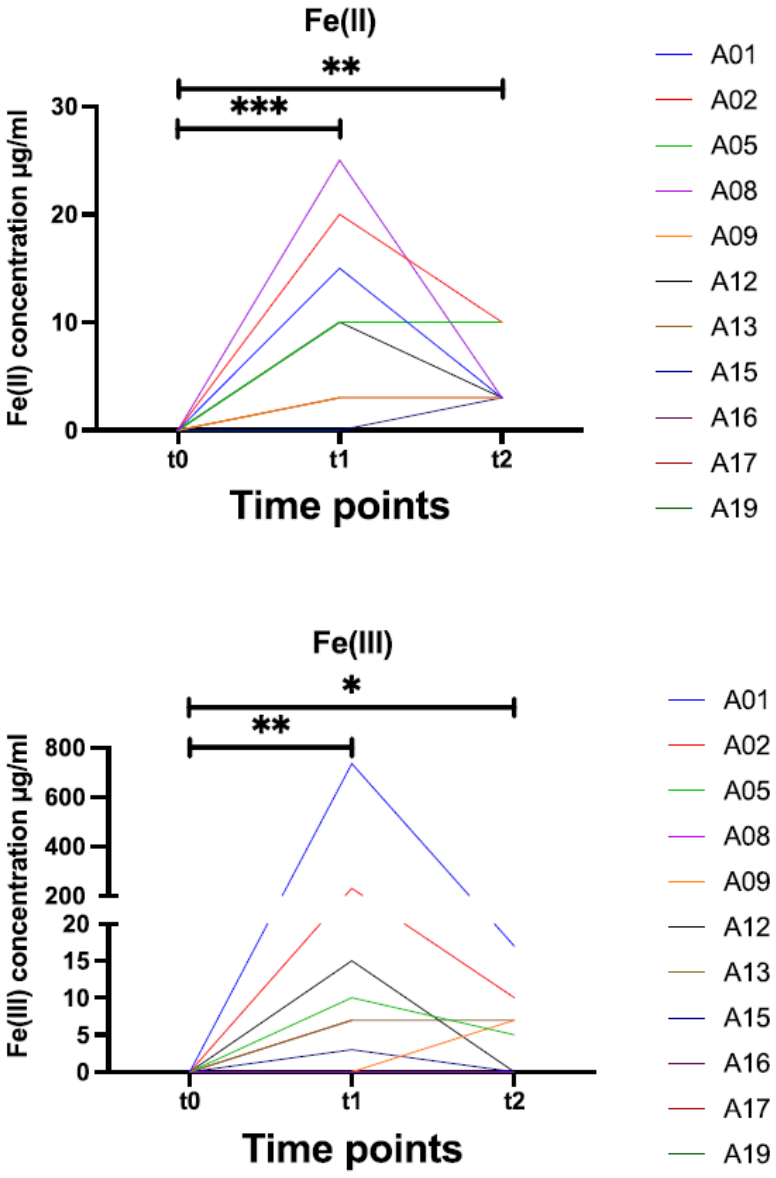

Figure 5. Dynamic of Fe(2+) and Fe(3+) detected at t0, t1 and t2 in the fecal supernatant of all 11 patients. The statistical analysis was performed using the non-parametric one-way ANOVA (Kruskal-Wallis) as non-pairing due to the missing measurements of patients A16, A17 and A19 at t2. The significance threshold was set at $p \leq .05$. For Fe(2+) ** = 0.0028 and *** = 0.0003; while for Fe(3+) * = 0.0424 and ** = 0.0085.