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The *In Situ* Human Dental Implantome: A First Appraisal

Abstract: Protein adsorption is the first fundamental interaction between the human body and a foreign surface. The sum of all proteins in this adherent proteinaceous layer comprises the implant proteome. The *in situ* dental implant proteome (implantome) was eluted from four implants of two wettabilities after a 2-min dipping in the *humor operationis* of maxillar tooth sockets. A mean number of 2056 different polypeptides per implant were identified according to the Xcorr method (Xcorr score ≥ 1.5, n ≥ 2 peptides). In the top 12 proteins comprising ca. 47% of the total abundance, cell-free hemoglobin (26.7%) was the most abundant, followed by fibrinogen (6.4%) and serum albumin (1.8%) with additional 1,800 lower abundance polypeptides, which contained ca. 34 salivary and a similar number of autoimmunogenic polypeptides. Selective enrichment of cell-free hemoglobin on the implant vs. albumin was estimated to 270 fold.

Keywords: implantomics, *humor operationis*, autoimmuno-implantomics, proteomics, biocompatibility, osseoinntegration

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1 Introduction

Even after ca. 35 years of research biocompatibility and osseointegration remain enigmatic [1,2]. Current teaching assumes, that the initial protein layer on implants consists only of proteins adsorbed from blood plasma. The plasma origin was disproven by the first human *in situ* proteome on hip implants two years ago [3]. The adsorbed proteins originate from the *humor operationis*, the operational socket fluid, consisting of up to 70% of intracellular proteins with the major protein being cell-free hemoglobin [3]. All present theories of osseointegration are based on the above false assumption of the plasma protein origin. The formation of the human implantome from *h. operationis* within seconds and minutes, is the first step of the foreign body reaction vs. an implant, initiating osseo-integration and subject of the new field of *implantomics* [4]. Here we report on a first clinical investigation of the human *in situ* intraoperative dental implant proteome.

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2 Materials and Methods

Implants. Dental implants (diameter: $\boldsymbol{D}$ = 3.3-3.4 mm) of differing wettability [5] (advancing dynamic contact angle, DCA_{ADV}) were purchased: three implants *type I* (Xive plus, 2x D3.4/L11 and 1x D3.4/L13) from Dentsply Sirona GmbH, D-64625 Bensheim with a hydrophobic surface (DCA θ_{Adv} ~105°–108°) and one implant *type II* (Logon® in.duce) D3.3/L13) from Logon/Nobel Biocare DE GmbH, D-52134 Herzogenrath with a hyperhydrophilic surface (DCA θ_{Adv} ~7i°–9i° [5] and a protective layer [6,7]. Sterile conditions were kept throughout. For surface concentrations ($\boldsymbol{\Gamma'}$) the "potential bone/implant contact areas" (pBICA, n= 6) of ref. [8] were averaged to a mean $\boldsymbol{D}$ = 3.3 ± 0.2 mm, mean length $\boldsymbol{L}$ = 10.3 ± 0.6 mm and mean pBICA = 1.37 ± 0.13 cm^2 [8]. pBICA corresponds to a "geometric surface area", under exclusion of micro and nano structures on "real surfaces" (see [9]).

Clinical settings. This clinical study consisted of two patients: patient 1 (68a; ♂; after sinus lift) and Patient 2 (77a; ♀; diabetes m., hypertension) allowing two sample implants each (n = 4) at the Implant Center Ruhr (Essen, DE), as endorsed by the University ethics commission (permit No. 19-9032-BO).

Table 1
Patient sample notations and implant assignments

	Implant 1	Implant 2	*Humor* 1	*Humor* 2
Patient 1	P1D16	P1D26	-	-
	Xive plus	Xive plus	-	-
Patient 2	P2D16	P2L24	P2H16	P2H24
	Xive plus	Logon	+	+

The two digit number corresponds to the FDI notation [10]. humor = humor operationis (see nomenclature below)

In situ patient dental implantome. Before final implantation in two patients sample implants were aseptically dipped in the fluid of drilled bone sockets ($\boldsymbol{D}$ = 3.8 mm; FDI maxillar positions "16", 26 & "24") [10], for two minutes *in situ* **(Table 1).** The retrieved implants carrying the

implantome were washed with saline (0.9% NaCl), quick frozen in liquid N_2 and stored at -80°C [3]. After thawing the whole implants below the cutting plane (i.e. pBICA) [8] were eluted in vials in three two-min steps of 0.5 ml each, under reducing conditions in 4% SDS with final addition of 0.3 ml idoacetamide buffer at ~25°C as described [3]. Protein was determined after TCA precipitation according to Lowry [3].

Humor operationis. Operational fluid samples of 800 -1000 µl, (10-20 mM EDTA, pH 7.4) were taken from patient 2 by syringe from two sockets (FDI positions "16" & "24"), prior to final implantation, kept on ice, centrifuged (8000 x g), quick frozen in liquid N_2 and stored at -80°C. For analysis at Mosaiques GmbH samples were denatured under reducing conditions with 4% SDS [3] and heated to ~100°C for 3 min.

In vitro plasma proteome. Citrated fresh frozen human blood plasma (-80°C), obtained from the Transfusion Medicine (UK-Essen), was thawed at 0°C and warmed to 37°C. After immersion of a Logon implant under slow movement in plasma for 2 min at 37°C, it was immersed in saline 1 min, then in fresh saline under whirling 1 min and finally rinsed with saline. It was fully eluted in a vial with 1.5 ml of 0.1 M NaOH, 4% SDS [11] for 5 min at 100°C and stored at -80°C.

Proteome analysis. Samples were analyzed by LC-MS/MS (Orbitrap Velos, Proteome Discoverer 1.4, Sequest FTMS) at Mosaiques GmbH, Hannover**.** From each cluster of the four samples the peptide sequence with the highest Xcorr score > 1.5 [12] was selected and applied for protein⌘ identification. In **Table 2,** in addition to the Xcorr ≥ 1.5 score, higher confidence levels, in the form of the false discovery rate approach (FDR 5% or 1%) [13,14], were employed leading to an exponential reduction in the number of proteins⌘. The four patient implantomes were then averaged by row-statistics (Prism 4, GraphPad, San Diego, CA, USA).

The term protein in proteomic nomenclature corresponds strictly only to a mature polypeptide chain, either as gene expressed or as post-translationally processed. Holoproteins such as hetero-oligomeric, quarternary structured proteins e.g. hemoglobin (two non-covalently bound subunits) or hetero-oligopeptidic disulfide crosslinked tertiary structured proteins e.g. fibrinogen (three covalently linked polypeptides) are officially listed as two or three separate entities respectively. The number of listed mature polypeptides is therefore greater than the actual number of holoproteins (see **Tables 4** and **8**). To avoid problems the annotated term protein⌘, denoting distinct "assumed proteins", will be used to indicate the identified polypeptides in proteomic analyses.

Nomenclature: The sample ID notation e.g. P1/D/16 corresponds to the *Patient No./implant company* (D: Dentsply, L: Logon)/and the *tooth position Numbers* 16, 26, 24 according to the international FDI terminology [10] (**Table 1**). All other terms used in this paper are defined in [4]**.**

3 Results and Discussion

3.1 General dental implantomics

Protein analysis of samples. The protein amount eluted after 2 min of adsorption from the four patient *in situ* implants is shown in **Table 2,** yielding a mean mass of m = 45.0 ± 6.9 µg/implant and a surface concentration Γ' = 32.7 ± 4.7 µg/cm^2. Protein multilayers appear possible. Similar values are eluted from the *in vitro* implant after plasma adsorption. The value of Γ' does not include the surface area increase from the micro and nano surface structures as considered in "actual surface" calculations [9]. Little is known on the DNA, RNA, lipid, carbohydrate or vesicle-compositions within the protein layer nor how the layer is removed e.g. by proteolysis or phagocytosis. In *humor operationis* (**Table 2**) the protein concentrations (53 mg/ml) are lower than in blood plasma.

Table 2
Protein in implant eluates (A) and humor operationis (B).[§]

A. Eluates	**Vol.** ml	**Conc.**	**mass**	**Γ',** µg/cm^2
Implants ***in situ***		**µg/ml**	**µg**	
P1D16	1.8	22	39.6	29
P1D26	1.8	26	46.8	34
P2D16	1.8	30	54.0	39
P2L24	1.8	22	39.6	29
Implant ***in vitro***				
2ADN	1.5	32	48.0	35
B. Humor operationis		**mg/ml**	**mg**	
P2H16	1.0	53	53	
P2H24	1.0	53	53	

[§]A mean pBICA = 1.37 ± 0.13 cm^2 (n=6) [8], i.e. a "geometric surface area" [9]), were used for calculating the surface concentrations (Γ'). In situ: implantome from socket fluid; in vitro: implantome from plasma. For further details see Methods.

Proteome analysis. The proteome analysis in the various fluids is in a sense still a "shotgun" situation [12]. As shown in **Table 3** different confidence levels for peptide identification and abundance of proteins⌘ exist, for which however a general consensus is still in evolution. As indicated in Methods three procedures were applied: Xcorr > 1.5; FDR ≤ 5%; and FDR ≤ 1%. An Xcorr > 1.5 [12], as employed in our previous work [3], allows compatibility with this work and inclusion of low abundance proteins⌘. Single peptides are excluded (**n ≥ 2)** in Xcorr but not in FDR. Increasing the confidence level to FDR exponentially reduces identified proteins⌘: For P1D16 in Xcorr we find 3330 peptides identifying 1884 proteins⌘. In FDR ≤ 5% only 235 peptides identify 196 proteins⌘:. In FDR ≤ 1% 189 peptides identify 165 proteins⌘ indicating only an

improvement of ca 15%. Since FDR ≤ 5% includes all in FDR ≤ 1% identified proteins⌘, it is used for both FDR levels here.

Table 3
Proteome analysis at three different confidence levels with numbers of analytical peptides and identified proteins⌘ §.

Samples	Identified and Quantified Proteins⌘					
	Xcorr > 1.5 n ≥ 2		**FDR ≤ 5%**		**FDR ≤ 1%**	
Patients	Peptides	Proteins⌘	Peptides	Proteins⌘	Peptides	Proteins⌘
P1D16	3506	1884	235	196	189	165
P1D26	4980	2216	339	251	274	212
P2D26	4985	2272	347	253	279	214
P2L24	3352	1855	233	197	191	169
Plasma						
2ADN	1502	505	189	131	156	120
Humor						
P2H16	1607	469	204	139	169	127
P2H24	2054	548	240	152	198	139

§*The term* proteins⌘ denotes assumed proteins.
For further details see Methods and the text..

Table 4
Top 12 proteins⌘ *of four averaged in situ dental implantomes*§

No.	Name	Symbol	Abundance [ppm]	
			mean	**±SE**
1	Hemoglobin (αβ subunits)*	HB(A,B)	192158	15978
2	Fibrinogen (αβγ chains)*	FG(A,B,C)	46287	7506
3	LisH domain-cont. protein*	FOPNL	17052	4535
4	Rho GTPase-activ. Prot. 21	ARHGAP21	16932	2748
5	Serum albumin*	ALB	13213	4984
6	Semaphorin-6A	SEMA6A	10869	2139
7	Spectrin β-chain, erythrocyte*	SPTB	10132	5394
8	Phospholip. phosphatase type 3	PLPPR3	8191	1178
9	Zinc finger protein 821	ZNF821	7905	4451
10	Putative exonuclease GOR	REXO1L1P	6753	1002
11	Titin	TTN	6398	2269
12	Protein PRR14L	PRR14L	5734	4985

§*Profile and abundance by Xcorr > 1.5 (n ≥ 2): mean ± SE (4), total ppm for 4 implants = 720907 ppm. *FDR ≤ 5%*

The mean data of the four patient implantomes are shown in **Table 4.** The proteins hemoglobin (= holoprotein of a 2x2-subunit structure: ($\alpha_2\beta_2$)) (26.7%), fibrinogen (= protein of 3x2 crosslinked polypeptides ($A\alpha,B\beta,\gamma)_2$) with 6.4% are the most abundant proteins plus serum albumin (monomer) with 1.8%. All 12 entries add up to an overall abundance of 47%.

3.2 Special dental Implantomics

Salivary proteins⌘. From 27 distinct protein groups in the implantome partly in the implantome two were selected. The first group contains at least 34 different salivary proteins⌘ (see [15,16]) including five protein⌘ families of lysozyme, galectins, mucins and proline-rich proteins shown in **Table 5**.

Table 5
Families of salivary proteins⌘ in the dental implantome §

Family	Name	Symbol	Abund., ppm	
			Mean	**SE**
1	Galectin-3*	LGALS3	3	1
	Galectin-7*	LGALS7	5	2
	Galectin-12	LGALS12	85	36
2	Lysozyme C*	LYZ*	13	10
3	Mucin-12	MUC12	136	63
	Mucin-16	MUC16	1479	228
	Mucin-19	MUC19	136	26
4	Proline-rich prot 12	PRR12	98	18
	Proline-rich prot 22	PRR22	366	106
	Proline-rich prot 25	PRR25	25	2
5	Protein S100-A4*	S100A4	9	3
	Protein S100-A6*	S100A6	35	8
	Protein S100-A9*	S100A9	17	2

§*For further details see **Table 4**; mean ± SE (4); *FDR ≤ 5%;*

Wettability and plasma (not shown). Implantome comparison of the hyperhydrophilic P2L24 vs. the hydrophobic implant P2D16 (**Table 3)** yields an Xcorr identity of the top 12 of 30%, and a 5-fold lower fibrinogen load (2.4% on P2L24 vs. 12.6% on P2D16. The plasma implantome (**Table 2**) is to be published.

Autoimmuno-implantomics. In the second group five protein families associated with autoantibodies [17,18] (**Table 6**) could be identified. It is indeed conceivable that such proteins⌘ can unfold on the implant surface exposing immunizing neo-epitopes as a cause of autoimmune diseases or even possibly of early (first year) implant failures, generally of unknown cause.

Table 6
Autoimmunogenic proteins⌘ in 4 averaged dental implantomes§

Family	Name	Symbol	Abund., ppm
			Mean ± SE
1	Cytochrome P450 20A1	CYP20A1	143 ± 107 (3)
	Cytochrome P450 2C8	CYP2C8	17 ± 7 (3)
	Cytochrome P450 2W1	CYP2W1	15 ± 15 (2)
2	Myeloperoxidase*	MPO	13 (1)
3	Heat shock protein 90 alpha*	HSP90AA1	46 ± 13 (4)
	Heat shock cognate 71 kDa*	HSPA8	18 ± 5 (4)
	heat shock protein 60 kDa*	HSPD1	62 ± 35 (4)
4	Disintegrin metalloprot. dom. 11	ADAM11	131 ± 42 (4)
	Disintegrin metalloprot. dom. 9	ADAM9	239 ± 144 (4)
	Disintegrin metalloprot. thrombo 20	ADAMTS20	53 ± 21 (3)
5	Collagen alpha-1(IV) chain	COL4A1	401 ± 72 (4)
	Collagen alpha-6(IV) chain	COL4A6	108 ± 43 (4)
	Collagen alpha-4(IV) chain	COL4A4	51 ± 18 (4)

§*For details see **Table 4** and [17,18], mean ± SE (n) *FDR <5%*

3.3 *Humor*-Proteomics

Table 6 shows the top proteins⌘ of the *h. operationis* proteome, from the socket holes for dipping the implants with different profile vs. **Table 4**. **Table 3** lists only ~ 500 proteins in *humor* instead of the ~ 2,000 in the implantome. This may be due to the high percentage of albumin in the mass spectra masking

other proteins⌘. From the ratio of HBB/ALB = 0.038 in *humor* (**Table 7**) and the ratio of HBB/ALB = 10.6 in implantomes (**Table 4)** the enrichment factor for the selective adsorption of hemoglobin on implants is estimated to be 279 fold.

Table 7
Top 12 proteins⌘ in averaged proteomes of humor operationis§

Nr	Name	Symbol	Abundance [ppm]	
			mean	SE
1	Serum albumin*	ALB	333568	54864
2	Immunoglobulin gamma-1 HC*	1SV	23217	4442
3	Alpha-2-macroglobulin*	A2M	20875	3292
4	Inner centromere protein	INCENP	20385	2907
5	Transmembrane protein 198*	TMEM198	16388	3534
6	Serotransferrin*	TF	12576	1399
7	Citron Rho-interacting kinase	CIT	12438	557
8	Hemoglobin subunit beta	HBB	11978	8471
9	Ubiquitin carboxyl-term. hydrol.*	USP7	11883	211
10	Rho GTPase-activating prot. 42	ARHGAP42	10762	1522
11	Zinc finger protein 40	HIVEP1	10634	61
12	Transforming growth factor-beta-induced protein ig-h3	TGFBI	10042	446

§*For further details see **Table 2**; mean ± SE (n = 2), *FDR <5%.*

3.4 Stoichiometry in Implantomics

The 1:1 subunit stoichiometry in hemoglobin and 1:1:1 Stoichiometry in fibrinogen are not reflected in 1:1 abundances in **Table 4.** As shown here in **Table 8** compared to a standard reference [19] hemoglobin lacks 60% of the physiological α subunit and fibrinogen 30% of the γ-chain. A similar unclear ratio of 0.3:1 is also found for hemoglobin in *humor* (**Table 7).**

Table 8
Apparent polypeptide stoichometry loss in Hb and FBG§

Polypeptide chains	ppm	Apparent Molar Ratio	Stoichiometry	
			[19]	Proteome
Hb α (m= 15.4 kDa)	52492	3.40	1.0	0.39
Hb β (m= 15.9 kDa)	139666	8.77	0.98	1.0
FBG Aα (m= 73 kDa)	17437	0.239	0.74	0.76
FBG Bβ (m= 60 kDa)	18775	0.313	1	1.0
FBG γ (M= 53 kDa)	10075	0.190	0.87	0.61

§*HB: hemoglobin; FBG: Fibrinogen; app. Molar Ratio: ppm/Da*

4 Conclusions

The key to biocompatibility and integration lies in the first protein layer on implants. This is the first report on the two-minute *in situ* determinations of the dental implantome and the corresponding *humor operationis*. The results indicate a wider clinical relevance due to detection of salivary, intracellular and especially autoimmunogenic proteins, some possibly unfolding on the implant surface with eventual release. Interface biology should now also include implantomic results, when focussing on the second, crucial human foreign-body reaction, i.e. the *in situ* cell behaviour and tissue development by and on biomaterials.

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