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Early risk prognosis of free-flap transplant failure by quantitation of the macrophage colony-stimulating factor in patient plasma using 2-dimensional liquid-chromatography multiple reaction monitoring-mass spectrometry

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Abstract

Although great success of microvascular free-flap transplantation surgery has been achieved in recent years, between 1.5% and 15% of flaps are still lost due to vascular occlusion. The clinical challenge remains to salvage a transplant in the case of vascular complications. Since flap loss is devastating for the patient, it is of utmost importance to detect signs of complications or of conspicuities as soon as possible. Rescue success rates highly depend on early revision. In this study, we collected blood samples during transplantation surgery from either the contributory artery or the effluent vein of the flap and applied a targeted mass spectrometry-based approach to quantify 24 acute phase proteins, cytokines, and growth factors in 63 plasma samples from 21 hospitalized patients, generating a dataset with 9450 protein concentration values. Biostatistical analyses of the targeted plasma protein concentrations in all 63 plasma samples showed that venous concentrations of macrophage colony-stimulating factor (M-CSF) provided the highest accuracy for discriminating patients with either clinical conspicuities or complications from control individuals. Using 21.33 ng/mL of M-CSF as the diagnostic threshold when analyzing venous blood plasma samples, the assay obtained a sensitivity of 0.93 and a specificity of 0.85 with an area under the curve value of 0.902 in the receiver operating characteristic analysis. Overall, our results indicate that M-CSF is a potential molecular marker for early risk prognosis of free-flap transplant failure.

Abbreviations: 2D = 2-dimensional, AUC = area under the curve, COCO = clinical conspicuity and complication, CTRL = control, FAS = full analysis set, FN = false negative, FP = false positive, MRM = multiple reaction monitoring, MS = mass spectrometry, NAT = natural, NPV = negative predictive value, PPS = per protocol set, PPV = positive predictive value, ROC = receiver operating characteristic, SIS = stable isotope-labeled standard.

Keywords: flap monitoring, free-flap surgery, I/R injury, macrophage colony-stimulating factor, mass spectrometry, microsurgical transplantation, multiple reaction monitoring (MRM), protein concentrations in plasma, relative quantitation, retrospective study

1. Introduction

Free-flap tissue transfer has become a reliable microsurgical procedure and is widely practiced by many medical centers, particularly in primary reconstruction following ablative breast surgery, replacement of soft tissue or bone structures, and covering of complex wounds on the surface of the human body. Despite the low failure rates of microvascular surgery in general, according to literature, up to 25% of the transferred tissues need to be revised due to complications that arise during the first days after surgery.[1–3] Loss of the transplanted tissue is devastating for the patient. Complications of free tissue transfers are mainly a result of vessel thrombosis that occurs either in the contributory artery or in the effluent vein. Since the venous system is a low flow/low pressure system, venous thrombosis occurs more often than arterial thrombosis.[4,5] Complications are divided into early onset (less than 30 days after surgery) and late onset complications (more than 30 days after surgery). Typical early complications include bleeding, thrombosis, infections, partial, or total flap loss, while late complications include seroma, fat necrosis, and infections.[6] In addition, procedures that deviate from standard processes during surgery caused by vascular irregularities without being a complication in the classical sense, such as the need of repeating vessel anastomosis or the need of a vessel graft, are addressed as clinical conspicuities. Lesions of the

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endothelial tissue of the vessel and stasis of blood flow are the main factors for thrombosis that may occur during surgery.[7]

Whenever such clinical conspicuities and/or complications occur, they must be identified as early as possible to allow for interventions to salvage the tissue. Although clinical techniques[8–19] are available for monitoring flap perfusion during postoperative periods, there is a lack of objective measures. Even for experienced staff the evaluation of the clinical situation often remains uncertain. In many cases complications are detected at a fairly late stage, leaving very little time to rescue a hypoxic flap. Late salvage procedures are associated with a high risk of flap failure.[20] Therefore, assessing the objective flap perfusion in the early postoperative phase is desirable, leaving more time for rescue intervention in case of complications.

During free-flap transfer, the transplant is disconnected from blood circulation (ischemia time) as long as the transplant is taken from the donor site and transferred to the recipient site. Reconstruction of blood flow is restored by anastomosis of at least 2 blood vessels for delivering blood from the recipient to the transplant and back.[21] After the time of ischemia, the ischemiareperfusion injury (I/R injury) follows – an inflammatory reaction that starts when the blood flow is restored.[22,23] During ischemia and upon reperfusion, both the tissue and residing peripheral blood cells release factors that trigger a series of molecular processes in the transferred flap, influencing success of the healing process. Macrophages and keratinocytes, for example, play a major role in the tissue regeneration processes as they release various proteins into the extracellular environment (i.e., into the blood plasma) which participate in clotting, in immune defense, and in tissue regeneration.[24–26]

Our method of searching for predictive marker proteins for both, clinical conspicuities and complications, was based upon the fact that ischemia and associated concurrent cellular damage causes release of acute phase proteins, cytokines, and growth factors into the circulation which provoke and initiate inflammatory responses and cellular healing processes, thereby influencing the clinical course of free-flap transplantation. Monitoring transplanted flap perfusion through molecular markers would therefore be expected to provide information at an early clinical stage enabling early prediction of conspicuities and/or complications and to increase the time for rescuing the transplant, if necessary. Human blood plasma contains tens of thousands of proteins which span over 10 orders of magnitude in concentration.[27] Fractionation of the proteolytic digest by 2 dimensions of reversed phase liquid chromatography separation – the 1st operated at high pH and the 2nd at low pH – has been shown to provide the orthogonality and sensitivity necessary to quantify low-abundance plasma proteins.[28,29]

Here, we report a retrospective study conducted with plasma samples from 21 patients who underwent free-flap transplantation. Blood was collected from each patient at 3 different time points directly at the recipient site (artery, vein 1, and vein 2) during surgery. Twenty-four proteins, belonging to the inflammatory, coagulation, and acute-phase protein groups were quantified in all samples by a 2-dimensional liquid chromatography/multiple reaction monitoring-mass spectrometry (2D LC/MRM-MS) approach.

2. Materials and methods

2.1. Donor blood collection, plasma preparation, and clinical course

This study was approved by the Ethics Review Board (KEK) of the Canton Zurich in Switzerland (ref. no. StV 8-2009). Blood was collected during surgery after written informed consent was obtained from all donors. Twenty-one patients who underwent free-flap transplantation surgery were recruited for this study (Table 1). All patients included were of Caucasian ethnicity. Inclusion criteria were good health and condition (ASA classification 1–2; https://www.asahq.org/), normal weight (BMI 20–25), non-diabetic, no manifest infection situation, and no essential diseases besides the main diagnosis. One arterial

Table 1

Clinical parameters, demographic data, and patient characteristics.

Clinical diagnosis	Patient ID	Gender	Age, year	Transplant flap type	Weight, g	Place of birth/current residency
CTRL	101	Male	62	Fibula	80	Canton Thurgau/Switzerland
	102	Male	54	Fibula	ND	Canton Aargau/Switzerland
	103	Male	55	Fibula	ND	Canton Zurich/Switzerland
	104	Female	66	Fibula	110	Serbia/Switzerland
	106	Male	58	Fibula	ND	Canton Zurich/Switzerland
	107	Female	57	Fibula	98	Canton Zurich/Switzerland
	201	Male	60	Latissimus dorsi	272	Finland/Switzerland
	204	Male	40	Serratus anterior	ND	Canton Ticino/Switzerland
	205	Male	62	Gracilis	ND	Canton Zurich/Switzerland
	206	Female	49	Latissimus dorsi	243	Canton Ticino/Switzerland
	301	Female	26	Radialis	14	Canton Appenzell/Switzerland
	303	Female	64	Radialis	20	Canton Zurich/Switzerland
	304	Male	69	Radialis	15	Canton Jura/Switzerland
	306	Female	37	Radialis	ND	Canton Ticino/Switzerland
COCO	105	Male	43	Fibula	72	Canton Ticino/Switzerland
	108	Female	67	Fibula	132	Canton Ticino/Switzerland
	109	Female	70	Fibula	64	Romania/Switzerland
	202	Female	66	Latissimus dorsi	198	Canton Thurgau/Switzerland
	203	Male	56	Latissimus dorsi	284	Canton Appenzell/Switzerland
	302	Female	51	Radialis	96	Canton Zurich/Switzerland
	305	Female	79	Radialis	ND	Croatia/Switzerland

COCO = clinical conspicuity and complication, CTRL = control, ND = not determined.

blood sample was taken intra-operatively (artery: prior to arterial anastomosis) from the donor vessel, and 2 venous blood samples (vein 1: directly after anastomosis; vein 2: 2 minutes after collecting vein 1 samples) were taken after arterial anastomosis from the effluent flap vein. For each patient, 3 blood samples (1.5 mL, each; artery, vein 1, and vein 2) were collected using S-Monovette Lithium Heparin syringes (Monovette, Sarstedt, Germany). Blood samples were immediately subjected to sedimentation of blood cells by centrifugation at 2000 × g at room temperature for 15 minutes. Plasma was sterile filtered (0.2 μm pore size; Sterifix, B. Braun Melsungen AG, Melsungen, Germany), divided into aliquots (100 μL, each), and stored at −80 °C prior to further analysis.[30]

2.2. Preparation of stable isotope-labeld standard (SIS) peptide mixtures

The panel was composed of 50 SIS peptides which were quantotypic for 24 plasma proteins. The average post-synthetic purity for the 50 SIS peptides was 93.5%, as revealed by capillary zone electrophoresis. In this study, an equimolar SIS peptide mixture (250 fmol/μL) was used. This was prepared from the combination of individual peptide stocks (14 μL, each at 100 pmol/μL) and 30% acetonitrile/0.1% formic acid (4.9 mL). The concentrated stock was stored as lyophilized aliquots at −80 °C until use.

2.3. Preparation of peptide mixtures from plasma samples

Following published protocols,[29–31] plasma samples (20 μL) were each diluted with 590 μL of 25 mM ammonium bicarbonate buffer (Sigma–Aldrich; St. Louis, MO), pH 8. Denaturation and reduction of proteins were done by adding 80 μL of 50 mM tris-(2-carboxyethyl)-phosphine (TCEP; Thermo Scientific; Rockford, IL), dissolved in 25 mM ammonium bicarbonate buffer, pH 8, and 80 μL of 10% sodium deoxycholate (Sigma–Aldrich) dissolved in ammonium bicarbonate buffer, pH 8. These solutions were incubated at 60 °C for 30 minutes. Alkylation was performed by adding 80 μL of 100 mM iodoacetamide (IAA; Sigma–Aldrich), dissolved in ammonium bicarbonate buffer, pH 8, to each of the samples. The solutions were kept at 37 °C for 30 minutes. Next, 80 μL of 100 mM dithiothreitol (DTT; Sigma–Aldrich), dissolved in ammonium bicarbonate buffer, pH 8, was added to each of the samples to quench any excess alkylating agent, and the mixtures were incubated at 37 °C for 30 minutes. Proteolytic digestions were performed by incubating the denatured and thiol-alkylated protein mixtures with 100 μL of 1 mg/mL sequencing-grade TPCK-treated trypsin (Worthington; Lakewood, NJ), dissolved in ammonium bicarbonate buffer, pH 8, at 37 °C for 16 hours. After quenching the proteolytic digestion with formic acid (180 μL at 3.6%), an aliquot of the equimolar SIS peptide mixture (100 μL at 100 fmol/μL) was added for normalizing the responses of the endogenous peptide targets. Precipitated deoxycholate was removed by centrifugation at 13,200 rpm for 10 minutes. The peptide-containing supernatants (1196 μL for each sample, equivalent to 934.38 μg protein) were loaded onto equilibrated Oasis Hydrophilic-Lipophilic-Balanced cartridges (10 mg sorbent; Waters; Milford, MA) for desalting, according to the manufacturer's protocol. Elution from the solid phase extraction cartridge was achieved with 600 μL of 55% acetonitrile in 0.1% formic acid (final pH 3). Each peptide mixture was lyophilized to dryness and stored at −80 °C.

2.4. 2D LC/MRM-MS instrumentation and measurement conditions

Immediately before 2D LC/MRM-MS analysis, each lyophilized peptide mixture was resolubilized by adding 1600 μL of 10 mM ammonium hydroxide solution, pH 10, for subsequent injection onto a high-pH reversed phase liquid chromatography-ultraviolet system.[29] High-pH RP-HPLC was performed using an XBridge BEH300 column (4.6 × 150 mm, 5 μm particles; Waters, MA) connected to a 1260 Infinity LC system (Agilent Technologies; Palo Alto, CA). The column and autosampler were kept at 40 and 4 °C, respectively. The fraction collector was kept at 6 °C. Separation was performed at a flow rate of 1 mL/minute over a 31-minute gradient. Mobile phases A and B were composed of 100% water and 100% acetonitrile, respectively. Mobile phase C (100 mM ammonium hydroxide, pH 10) was held constant at 10% of the total solvent volume. The gradient for high-pH RP-HPLC separation was based on the previously described protocol[29,32] and set as follows (time in minutes, % mobile phase B solvent volume): 0,3; 3,3; 3.05,6.5; 4,6.5; 4.05,10; 5,10; 5.05,13.5; 6,13.5; 6.05,17; 7,17; 7.05,20.5; 8,20.5; 8.05,24; 9,24; 9.05,27.5; 10,27.5; 10.5,31; 11,31; 11.05,36; 12,36; 12.05,41; 13,41; 13.05,46, 14,46; 14.05,51; 20,51; 21,80; 30,80; and 31,3. For each sample, 47 fractions (approximately 500 μL, each) were collected into a 96-well plate (Axygen; Union City, CA). Each run was followed by a 14-minute isocratic run at 3% B, 87% A, and 0% C for column reequilibration. High-pH fractions were lyophilized to dryness (SuperModulyo freeze dryer, Thermo Scientific) and stored frozen at −80 °C. The high-pH RP-HPLC fractions were rehydrated. Fractions were pooled at defined intervals according to a previously developed protocol,[29] yielding 13 fractions with a final volume of approximately 100 μL each for each digested patient plasma sample. After transferring each of the 13 fractions into an injection vial, 20 μL of each fraction was loaded onto a 1290 Infinity LC system (Agilent Technologies) and the peptides were separated using an RP-HPLC column (Zorbax Eclipse Plus C18 Rapid Resolution HD, 2.1 × 150 mm, 1.8 μm particles; part no. 959759-902; Agilent Technologies) at a flow rate of 0.4 mL/minute over a 20-minute gradient (3%–81% mobile phase B). The compositions of the mobile phases were 0.1% formic acid in water for A, and 0.1% formic acid in acetonitrile for B. The optimized gradient for this panel of peptides was (time in minutes, % mobile phase B) 0,3; 1,8; 5,14.5; 6.5,16.5; 13,20.7; 16,24; 16.5,27; 17,81; 18,81; 19.9,81; and 20,3. Eluates from the LC were directly introduced into an Agilent 6490 QqQ mass spectrometer which was operated in the positive ion mode. The MRM acquisition parameters were set as follow: 3.5 kV capillary voltage, 300 V nozzle voltage, 11 L/minute sheath gas flow at 250 °C, 15 L/minute drying gas flow at 150 °C, 30 psi nebulizer gas flow, 380 V fragmentation voltage, and 5 V cell accelerator potential.[32–34] Three hundred transitions (i.e., 50 peptides with 3 transitions per peptide form) were targeted in each of the LC fractions with dwell times of 10.17 ms and cycle times of 700 ms. A 3-minute post-column equilibration time was performed after each sample analysis, and a blank injection of 0.1% formic acid was run between each sample analysis to reduce carryover. A daily human plasma quality control (QC) kit (catalog no. LCMSP-D-A6490, Cambridge Isotope Laboratories; Andover, MA) was used for performance evaluation. The lyophilized mix was rehydrated, as per the supplied protocol, then run at defined points throughout the analysis.

Table 2

M-CSF concentrations in vein 2 samples and J max determination.

Patient ID	Clinical diagnosis	ng/mL*	Test cut-off	Below cut-off	Above cut-off	TP	FP	FN	TN	Sensitivity	Specificity	J†
		10.45										
201	CTRL	11.45	10.95	0	21	7	14	0	0	1.00	0.00	0.00
103	CTRL	12.94	12.19	1	20	7	13	0	1	1.00	0.07	0.07
206	CTRL	13.69	13.32	2	19	7	12	0	2	1.00	0.14	0.14
204	CTRL	13.76	13.73	3	18	7	11	0	3	1.00	0.21	0.21
301	CTRL	14.96	14.36	4	17	7	10	0	4	1.00	0.29	0.29
304	CTRL	16.68	15.82	5	16	7	9	0	5	1.00	0.36	0.36
106	CTRL	16.97	16.82	6	15	7	8	0	6	1.00	0.43	0.43
104	CTRL	17.86	17.41	7	14	7	7	0	7	1.00	0.50	0.50
108	COCO	18.66	18.26	8	13	7	6	0	8	1.00	0.57	0.57
101	CTRL	19.60	19.13	9	12	6	6	1	8	0.86	0.57	0.43
102	CTRL	20.05	19.83	10	11	6	5	1	9	0.86	0.64	0.50
306	CTRL	20.92	20.49	11	10	6	4	1	10	0.86	0.71	0.57
303	CTRL	21.05	20.98	12	9	6	3	1	11	0.86	0.79	0.65
109	COCO	21.61	**21.33**	**13**	**8**	**6**	**2**	**1**	**12**	**0.86**	**0.86**	**0.72**
107	CTRL	22.68	22.14	14	7	5	2	2	12	0.71	0.86	0.57
105	COCO	24.08	23.38	15	6	5	1	2	13	0.71	0.93	0.64
205	CTRL	27.39	25.73	16	5	4	1	3	13	0.57	0.93	0.50
305	COCO	28.52	27.96	17	4	4	0	3	14	0.57	1.00	0.57
302	COCO	29.33	28.93	18	3	3	0	4	14	0.43	1.00	0.43
202 (1)	COCO	37.10	33.22	19	2	2	0	5	14	0.29	1.00	0.29
202 (2)	COCO	39.84	38.47	20	1	1	0	6	14	0.14	1.00	0.14
		40.84	40.34	21	0	0	0	7	14	0.00	1.00	0.00

COCO = clinical conspicuity and complication, CTRL = control, FN = false negative, FP = false positive, M-CSF = macrophage colony-stimulating factor, TN = true negative, TP = true positive.

* Ranking according to M-CSF protein concentration after adjoining theoretical minimum and maximum values.

† J = Youden index.

2.5. MRM data processing and cleanup

Raw MRM data were analyzed using the Agile2 integrator algorithm in the MassHunter Quantitative Analysis software (version B.07.00; Agilent Technologies, CA) using the Agilent integrator algorithm for peak integration. For each peptide transition, the accuracy of the peak selection and integration were determined based on retention time, peak width, and signal response. The "best" interference-free transition of one "natural" (NAT) peptide, that is, the one with the most intense MRM signal, was used for protein quantitation. Interference assessment was performed in plasma through signal-intensity ratio measurements on the NAT and SIS peptide transitions. Protein concentrations in ng/mL were calculated by taking into account the protein's molecular weight (in g/mol; obtained from ExPASy's "pI/Mw tool" [http://web.expasy.org/compute_pi/]), the peptide's relative response (NAT/SIS), the corrected SIS peptide concentration (i.e., corrected with the composition and the purity values, as determined by amino acid analysis and capillary zone electrophoresis), and a conversion factor (of 1000).[35] In cases where multiple peptides were quantified for a given protein, the one that provided the highest protein concentration was used as the quantifier.

2.6. Biostatistical analyses

To determine which sample time point and which protein concentration should be used to classify a patient as either belonging to the conspicuity/complication (clinical conspicuity and complication [COCO]) or control (CTRL) group, a Youden index analysis was performed.[36] First, all patient samples that belonged to 1 sampling time point were ranked according to their experimentally determined concentrations of a specific protein, for example, macrophage colony-stimulating factor (M-CSF) (Table 2). Then 2 theoretical concentration values were added. The 1st theoretical concentration value was determined by subtracting the value "1" from the lowest concentration and the 2nd by adding the value "1" to the highest concentration. These values were added at the top and at the bottom of the protein concentration list. Next, linear interpolation[37] between each pair of 2 neighboring concentration values was used to calculate the "test cut-off" values. With each "test cut-off" value it was then determined how many of the samples had protein concentration values below the "test cut-off" value and how many had protein concentration values above that value. Then it was determined which of the samples below the "test cut-off" were true negatives and which of them were false negatives (FN) by labeling the samples with the "gold standard" clinical assessment data. Similarly it was determined which of the samples that were above the "test cut-off" were true positives or and which were false positives (FPs). Next, sensitivity and specificity[38] were calculated for each of these biostatistical estimations at each "test cut-off" point. In addition, at each "test cut-off" value, the Youden index (J = sensitivity + specificity − 1) was determined.[36] The highest J value (J max) in the list of samples determined the best discrimination threshold concentration, that is, the best "cut-off point" within the samples within this test set. This procedure was repeated for all 21 proteins that passed the quality assessment, and for all the sampling time points (artery, vein 1, and vein 2). Ultimately, this generated 63 tables, in each of which the J max value for the respective test set was indicated (cf Table 3, Supplemental Tables 2 and 3, http://links.lww.com/MD/B287). To select the "optimal cut-off" value for discriminating "COCO" from "CTRL" samples irrespective of the sampling time point,[37] all J max values were ranked. Sensitivity and specificity as well as receiver operating characteristics (ROCs) and Youden indices[39,40] were calculated using the

Table 3

Best cut-off values and areas under the curves for 21 proteins from 21 vein 2 plasma samples[*].

Protein name	Best cut-off, ng/mL[†]	Sensitivity[‡]	1-Specificity[‡]	J max[§]	ROC (AUC)
Macrophage colony-stimulating factor 1	21.33	0.86	0.14	0.71	0.908
Intercellular adhesion molecule 1	218.14	1.00	0.50	0.50	0.786
Cathepsin D	76.16	0.86	0.50	0.36	0.663
Cathepsin B	16.84	0.86	0.36	0.50	0.643
Vascular cell adhesion protein 1	950.23	0.43	0.07	0.36	0.633
Retinol-binding protein 4	23048.69	0.29	0.00	0.29	0.592
Apolipoprotein A-I	3047873.48	0.29	0.00	0.29	0.582
Apolipoprotein C-III	406.64	0.71	0.43	0.29	0.582
Tissue factor pathway inhibitor	21.90	0.71	0.43	0.29	0.582
CD44 antigen	3091.36	0.43	0.00	0.43	0.561
Platelet-activating factor acetylhydrolase	124.42	0.71	0.29	0.43	0.561
Vitamin K-dependent protein C	1172.10	0.57	0.36	0.21	0.480
Plasminogen	149705.42	0.86	0.71	0.14	0.469
Complement C3	1182636.72	0.29	0.00	0.29	0.449
Interleukin-1 receptor-like 1	18.15	0.43	0.29	0.14	0.449
Protein S100-A8	1.85	0.71	0.64	0.07	0.439
Complement C5	59621.42	0.14	0.00	0.14	0.408
C-reactive protein	125.88	1.00	0.79	0.21	0.408
Osteopontin	18.81	0.43	0.36	0.07	0.367
Serum amyloid A-1 protein	89.02	1.00	0.86	0.14	0.296
Serum amyloid A-4 protein	9917.11	1.00	1.00	0.00	0.245

AUC = area under the curve, ROC = receiver operating characteristic.
[*] Ranking according to ROC.
[†] Optimal cut-off concentration at J max.
[‡] Calculated from individual concentrations of all 21 proteins.
[§] J = Youden index.

IBM statistics software SPSS (version 20.0, SPSS Inc, Chicago, IL). Logistic regression analysis[41] was performed using the Origin statistics software (version. 8.1 G; Originlab Corporation, Northampton, MA). Power analysis was conducted using the G*Power software (version 3.1, University of Düsseldorf).[42] Input parameters for power analysis of the combined vein 1 plus vein 2 groups were set as follows: the required power (1-β error probability) was 0.90; the allocation ratio N2/N1 was 0.5. Other parameters were left as default values.

3. Results

3.1. Demographic data, blood collection, and clinical assessment

The 63 arterial and venous blood samples were collected from 11 female and 10 male patients. Depending on the wound type of each patient (Table 1), the transferred tissue included bone (fibula flap), muscle (latissimus dorsi, serratus anterior, or gracilis muscle flap), or skin (radialis flap).

During and after microvascular flap transplantation, 7 patients showed clinical conspicuities or complications (COCO group). All conspicuities and complications in our study were early onset complications, so the direct causes could be traced back to surgery in every case. One patient (#202) developed a partial flap necrosis during the first 2 weeks after surgery and needed 1 revision operation. During this operation the necrotic tissue was removed and the flap was mobilized, so the defect could be closed with the remaining of the transplanted tissue. Another patient (#108) developed local flap infection in the 1st week after surgery, so 1 revision operation with cleaning and drainage of the wound was necessary; the further clinical course was uneventful. Patient #302 developed hematoma at the recipient site a few hours after surgery, so a revision operation was necessary to stop bleeding and to evacuate hematoma. The remaining 4 patients from our COCO group showed vascular conspicuities. In 3 cases (patients #109, #203, and #305), an intra-operative thrombosis of one anastomosis occurred, thereupon thrombectomy and revision anastomosis of the vessel had to be done and in patient #109 an interposition of a vein graft became necessary. Patient #105 was detected as clinically conspicuous as well with a low flap outflow through the vein after anastomosis which improved spontaneously. Of note, this patient's transplant experienced an extended ischemia time of 150 minutes.

Noteworthy, patients with ischemia times longer than 120 minutes were patients #103, #105, #109, and #302. Among these, patient #103 was the only one who did not develop a complication, confirming that long operation duration and ischemia time are important factors for developing complications. Fourteen patients (CTRL group) were free of distinctive features or complications during healing.

Clinical monitoring of the transplanted tissue included observing the color, the swelling, and the skin texture of the flap. A change in color could indicate a venous congestion or arterial lack of perfusion, for example, due to thrombosis (cf Fig. 1). Such clinical signs require instant revision operations to salvage the flap.

Since both situations – conspicuities and complications – are of concern during microvascular surgery, we combined them into 1 group (COCO group) and matched these patients to those in the group of CTRLs.

3.2. Protein concentration determination in plasma samples

Quantitation of 24 proteins in 63 plasma samples was accomplished using a bottom-up 2D LC-MRM/MS approach with SIS peptides serving as internal standards. This "full analysis set" (FAS) comprised 9450 protein concentrations (i.e., 50

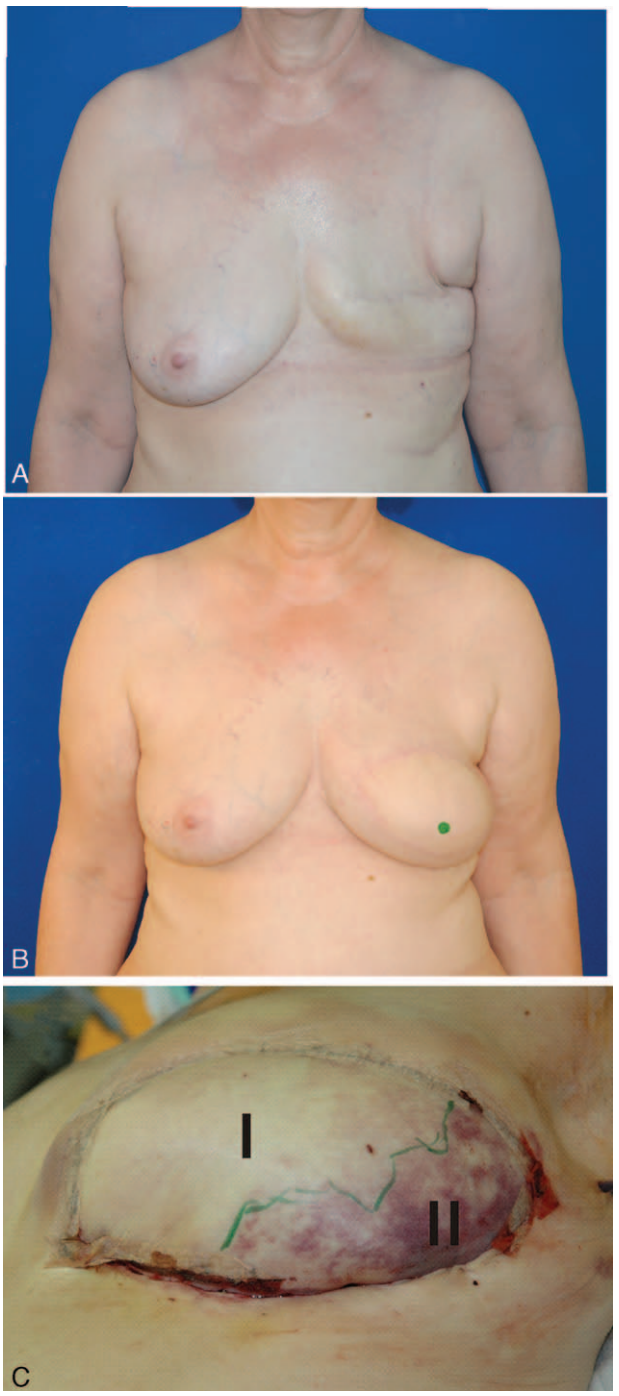

Figure 1. Exemplary cases for free tissue transfer. (A) Patient after amputation of the left breast. (B) Same patient 6 months after the 1st step of microvascular breast reconstruction with a transplant of skin and fatty tissue from the belly (deep inferior epigastric artery perforator [DIEP] flap). The green spot marked on the left breast represents the position of the later nipple-areola complex that will be reconstructed in the 2nd and last step of breast reconstruction. (C) Another patient after DIEP flap transplantation. In the area marked with (I) the flap develops good, in region (II) there is a lack of perfusion. There is an intermediate zone in between (green).

peptides × 3 relative ratios per peptide × 63 samples). The proteins were selected from previous quantitation panels representing inflammatory, coagulation, and acute-phase protein groups. During data cleanup and quality inspection, 1 quantifier peptide and transition was selected for each target protein. Data for 3 proteins (endoglin, P17813; serine/threonine-protein kinase MAK, Q547D0; and Nurim, B0S7R0) did not meet our required quality criteria (e.g., their concentrations could only be determined in a small number of samples), and were therefore excluded from further analysis.

After removing the missing data and redundancies, the "per protocol set (PPS)" contained 1302 protein concentration values (21 proteins × 62 plasma samples; see Supplemental Table 1, http://links.lww.com/MD/B286) which was followed by biostatistical data analysis. In our PPS, the protein concentration of the most abundant protein was 3.7 mg/mL for apolipoprotein A-I (in sample 102, sampling time point Artery), while that of the least abundant protein was 0.88 ng/mL for protein S100 A8 (in sample 102, sampling time point vein 2). The determined protein concentrations that were routinely quantified by 2D LC/MRM-MS assays spanned over 5 orders of magnitude.

3.3. Biostatistic data analysis and determination of risk prognosis marker

It should be noted that at the time point of blood sampling, that is, during surgery, the clinical outcome was not known. By the time of statistical analysis, however, a clinical assessment had been given and was recorded as the true status of the patient, the "gold standard," because by this time it was known whether or not the patient who donated the sample had experienced complications during wound healing, that is, after transplantation surgery (Table 1). Biostatistical evaluation focused on the search for the best discriminating protein to predict patients that would experience conspicuities and/or complications or would fall into the CTRL group.

For example, the average M-CSF concentration (mean ± SD) was 17.86 ± 4.40 ng/mL for the CTRL group and 28.45 ± 7.82 ng/mL for the COCO group with vein 2 samples. The calculation process for M-CSF (Table 2) revealed a J max value of 0.72 which was obtained with the best "cut-off point" – an M-CSF concentration of 21.33 ng/mL.

By calculating J max values for all 21 proteins separately for each of the 3 sampling time points (artery, vein 1, and vein 2), 63 "best cut-off points" were generated in total. In parallel, for all 21 proteins with each sampling time point, a total of 63 ROC curves with the corresponding area under the curve (AUC) values were calculated and compared to each other. At the "best cut-off

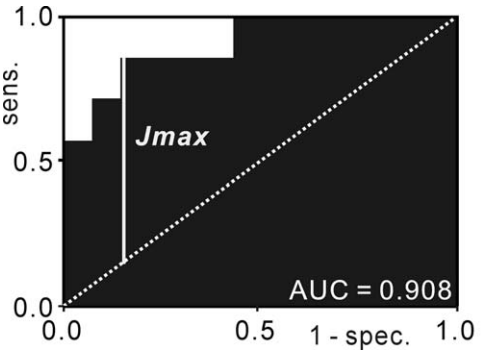

Figure 2. ROC analysis of plasma M-CSF concentrations from vein 2 samples. Calculated AUC is 0.908. The vertical solid line indicates the maximum Youden index (J max), the hatched diagonal indicates the 0.5 value. AUC = area under the curve, M-CSF = macrophage colony-stimulating factor, ROC = receiver operating characteristic.

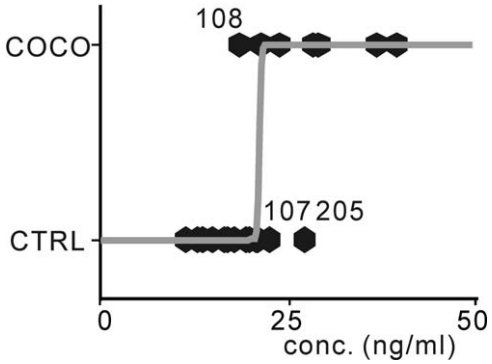

Figure 3. Logistic regression analysis of plasma M-CSF concentrations from the vein 2 group. COCO (N = 7) and CTRL (N = 14). Outliers are labeled with patient ID numbers. CTRL = control, M-CSF = macrophage colony-stimulating factor.

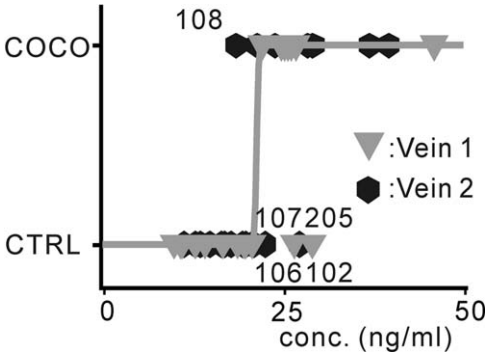

Figure 4. Logistic regression analysis of plasma M-CSF concentrations from the combined vein 1 plus vein 2 groups. COCO (N = 14) and CTRL (N = 27). Outliers are labeled with patient ID numbers. CTRL = control, M-CSF = macrophage colony-stimulating factor.

point" for M-CSF in the vein 2 group, the AUC was 0.908 (Table 3, Fig. 2).

Interestingly, M-CSF showed the best AUC values at all 3 sampling time points out of the 21 proteins whose protein concentrations were determined in all the samples. AUC values for M-CSF were 0.760 with the artery sampling time point and 0.879 with the vein 1 sampling time point, respectively (Supplemental Tables 2 and 3, http://links.lww.com/MD/B287).

The M-CSF concentration that was determined as "best cut-off point" with the vein 1 sampling time point was 21.34 ng/mL and was nearly the same as that for the vein 2 sampling time point (21.33 ng/mL). In contrast, with the artery sampling time point, the "best cut-off point" was 15.42 ng/mL of M-CSF. It should be noted that artery blood represented the global situation of the host, whereas venous blood represented the situation of the flap during surgery. Thus, focusing on the venous samples seemed to be preferable.

For all these "best cut-off points," the "optimal cut-off point" was determined, that is, the discrimination concentration at which the best separation of samples was achieved according to clinical assessment. Of all of the 21 proteins belonging to inflammatory, coagulation, and acute-phase proteins, the concentration of M-CSF in venous samples (vein 1 or vein 2) showed the greatest accuracy in discriminating conspicuities or complications from CTRL samples.

3.4. Evaluation of M-CSF as risk prognosis marker

To evaluate the effectiveness of a discrimination test that is based on venous M-CSF plasma concentrations, we performed logistic regression analysis using the individual M-CSF plasma concentrations. With vein 2 samples and an M-CSF concentration of 21.33 ng/mL as the "optimal cut-off point," 18 of the 21 patient samples were mapped to their correct classifications (Fig. 3). Six out of the 7 samples from patients with conspicuities during operation or complications were correctly placed into the COCO class, leaving just 1 FN case (#108). Patient 108 was the only patient with an infection as complication, whereas the other conspicuous patients from the COCO group developed conspicuities in the vascular system. Similarly, 12 out of the 14 CTRL patients were correctly placed in the CTRL class, leaving 2 FP cases (#107 and #205).

When the samples from both sampling time points, vein 1 and vein 2, were combined (Fig. 4), the number of true-positive cases was 13, leaving 1 FN case (vein 2: #108) and the number of true-negative cases was 23, leaving 4 FP cases (vein 1: #106 and #102; vein 2: #107 and #205) when applying the logistic regression analysis with an M-CSF concentration of 21.33 ng/mL as the "optimal cut-off point."

Biostatistical evaluation of patient stratification based on M-CSF plasma concentrations (Table 4) showed positive predictive values of 0.78 and 0.75, and negative predictive values of 1.00 and 0.92 for vein 1 and vein 2, respectively.

Combining the datasets from the 2 venous blood sample groups gave comparable results. When the "optimal cut-off point" of 21.33 ng/mL of M-CSF (Table 4) was applied for both vein groups, the ROC analyses (Fig. 5) of the combined "vein 1 plus vein 2" group resulted in an AUC of 0.902, with a sensitivity of 0.93 and a specificity of 0.85, suggesting good assay performance.

To exclude confounding results, that is, to verify that M-CSF concentrations were not associated with clinical parameters, we performed statistical comparisons (2-sample t tests and linear regression analyses) between the M-CSF plasma concentrations from the vein 1 group and metric clinical parameters but found no significant correlation neither between patient age and M-CSF plasma concentrations ($R^2 = 0.04$, $P = 0.21$), nor between flap weight and M-CSF plasma concentrations ($R^2 = 0.007$, $P = 0.31$). Similarly, no significant differences were found in M-CSF plasma

Table 4

Biostatistical evaluation of patient stratification*.

Sampling time point	TP	FP	TN	FN	Sensitivity	Specificity	FP rate	FN rate	Pos. pred. value	Neg. pred. value	ROC AUC
V1	7	2	11	0	1.00	0.85	0.15	0.00	0.78	1.00	0.879
V2	6	2	12	1	0.86	0.86	0.14	0.14	0.75	0.92	0.908
V1 + V2	13	4	23	1	0.93	0.85	0.17	0.07	0.76	0.95	0.902

AUC = area under the curve, FN = false negative, FP = false positive, M-CSF = macrophage colony-stimulating factor, ROC = receiver operating characteristic, TN = true negative, TP = true positive.
* Based on M-CSF plasma concentrations.

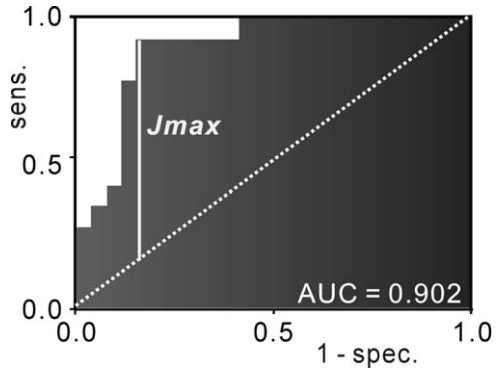

Figure 5. ROC analysis of plasma M-CSF concentrations from combined vein 1 plus vein 2 samples. Calculated AUC is 0.902. The vertical solid line indicates the maximum Youden index (J max), the hatched diagonal indicates the 0.5 value. AUC = area under the curve, M-CSF = macrophage colony-stimulating factor, ROC = receiver operating characteristic.

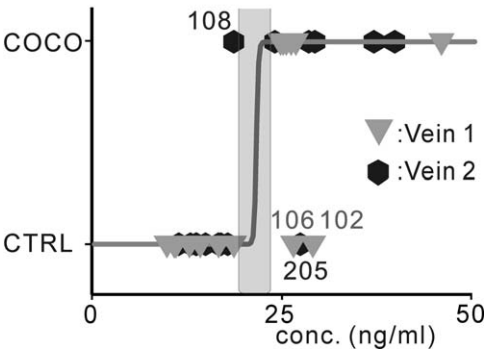

Figure 6. Logistic regression analysis of plasma M-CSF concentrations. Plasma samples from the combined vein 1 plus vein 2 groups. COCO (N = 14) and CTRL (N = 27). Samples that fall within the safety margins (shaded area; 19.3–23.4 ng/mL) are excluded. CTRL = control, M-CSF = macrophage colony-stimulating factor.

concentration distributions neither between genders ($P = 0.32$) nor between transplant tissue types ($P > 0.1$ in all 3 comparisons).

Power analysis with M-CSF protein concentrations of the combined vein groups (vein 1 plus vein 2) indicated that the minimally required sample size was 32, which was smaller than the actual number of 41 samples. Additionally, the calculated actual power of 0.96 suggested that the test was confidently representing statistical significance in discriminating conspicuities or complications from CTRLs, despite the rather small number of patients in our study.

To our knowledge this is the first report that correlates M-CSF protein levels in plasma with complications that arise during the healing process of free-flap transplants.

4. Discussion

2D LC/MRM-MS was found ideal for protein quantification of clinical samples because of its multiplexing potential (253 proteins from >600 interference-free peptides), high specificity (each targeted peptide is unique, and without interference from other peptides), high sensitivity (lower limit of quantification [LLOQ] in the pg/mL range), low sample consumption (20 μL raw plasma), and without the need for antibodies or other means of depletion or enrichment.[29,31] Interestingly, previously published venous M-CSF concentrations from healthy volunteers in which 2D LC/MRM-MS analysis was applied reported an average protein concentration of 16.05 ng/mL in plasma samples,[29] which is quite close to the average concentration of 16.93 ng/mL (±6.14 ng/mL) in vein 1 samples from CTRL individuals, that is, patients without complications during wound healing, indicating that this method is highly reproducible and reliable. Additionally, the average M-CSF concentration in the donor samples was determined be to 24 ng/mL by spectral counting, which also corresponds to the average protein concentration determined in a previous study.[43]

M-CSF is a hematopoietic growth factor[44,45] that is involved in proliferation, differentiation, and survival of the monocyte/macrophage lineage.[46–48] A variety of cell types including macrophages, endothelial cells, fibroblasts, lymphocytes, and bone marrow-derived stromal cells produce M-CSF.[49,50] Macrophages are the cells that predominantly become activated by M-CSF, after which they release a multitude of mediators that regulate inflammation and tissue repair.[51] It has been shown that macrophages exert their influence in both acute kidney injury and chronic renal failure.[52,53] Along with our findings, production of Th1 cytokines, that is, IFN-γ, were considered to recruit macrophages into kidney grafts, where activated macrophages showed enhanced cytotoxic T cell (CTL) activation, amplifying an ongoing immune response by upregulating the expression of both, major histocompatibility complex (MHC), and co-stimulating molecules on graft parenchymal cells and APCs.[54] Blockage of M-CSF reduced macrophage proliferation and accumulation in renal allograft rejection.[55] M-CSF serum levels significantly increased in acute rejection patients.[56] In our study, venous plasma levels of M-CSF were significantly elevated in the COCO group as compared to the CTRL group, which is in agreement with these reports. Interestingly, in our study, increased levels of M-CSF did not necessarily match with trauma history or long preoperative history, for example, wound conditioning.

Considering potential future clinical application of M-CSF concentrations as predictive marker for higher or lower risk of transplant failure, a ±10% tolerance interval was suggested to be considered above and below the "optimal cut-off point."[57] These "safety margins" generate a zone with width of 4.1 ng/mL of M-CSF between 19.3 and 23.4 ng/mL in plasma. Hence, instead of only assigning 2 groups, below or above the "cut-off point," a 3rd group can be defined whose concentrations fall within the safety margins. From our venous blood plasma samples this was true for 10 samples (103V1, 105V1, 205V1, 303V1, 101V2, 102V2, 107V2, 109V2, 303V2, and 306V2). For these, a statement about the risk of healing complications might not be given. By contrast, for the remaining 31 samples, risk assessment was provided with higher confidence by applying logistic regression analysis (Fig. 6).

Considering the safety margins, the positive predictive values were calculated as 0.75, 0.83, and 0.79 and negative predictive values of 1.00, 0.89, and 0.94 for vein 1, vein 2, and the combined venous samples, respectively (Supplemental Table 4, http://links.lww.com/MD/B287). In addition, the ROC (AUC) values for vein 1, vein 2, and the combined venous sample dataset (V1 plus V2) were all comparable to each other and reached excellent values, that is, above 0.9.[38]

Power analysis with the 31 remaining vein samples that were located outside of the safety margins indicated that the minimally required sample size was 24 in this setting. Again, the calculated actual power of 0.96 suggested that the test was confidently

representing statistical significance in discriminating the 2 groups for which prediction of the flap failure risk was made. Still, the number of patients of this retrospective study is considered comparably small.

The only "false negative" patient (#108) in our study experienced an infection 1 week after surgery, explaining the low M-CSF levels in the investigated blood samples, as these were obtained earlier during surgery. Such cases of "late ascents" of M-CSF concentrations will not be detected by the here introduced assay which may be regarded a weakness of our method. Yet, in this context, it should be mentioned that an assay for predicting healing success which produces higher numbers of "false negatives" is more problematic than an assay that produces higher numbers of "false positives," since for "false positives," clinical inspection will be done with higher alertness and "false alarms" do not physically harm the patient. Based on our results, we suggest that patients with M-CSF concentrations around and above the "optimal cut-off point" should be carefully evaluated and monitored during the post-operative period. For this intention, it would be interesting to also investigate M-CSF concentrations in the peripheral blood.

Also of clinical importance is the finding that there is no specific time point at which sampling of venous blood had to be done during surgery; at least within the short time frame that was investigated in this study. Our results further suggest that there is no need to collect artery blood for flap quality assessment.

From our results it is tempting to speculate that medical intervention by which M-CSF levels are modified might be considered as accompanying or even preventive treatment for free-flap transplantation surgery. Suitable medication may make use of either M-CSF-targeting antibodies or M-CSF receptor-targeting antibodies which are already in use or are being tested in clinical studies for other indications.[58–60] In conjunction with free-flap transplantation, anti-M-CSF treatment may be advised after determination of individual M-CSF concentrations once levels are found above threshold. It remains to be seen in future clinical trials whether a positive effect on free-flap perfusion and reduced cases of complications can be achieved as outcome of medication-based intervention.

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