Chapter 6

Salvaged blood and cytokine gene expression after hip surgery

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Submitted

ABSTRACT

Background: In orthopaedic surgery, re-infusion of autologous salvaged blood can be used as an alternative for allogeneic blood transfusion. In addition, autologous salvaged blood might stimulate an immune response reducing infections in the postoperative period.

Questions/purposes: We investigated whether re-infusion of washed or unwashed salvaged blood resulted in different cytokine gene expression (GE) profiles, indicative for immunomodulation, in patients after hip replacement surgery compared to the effect of the surgery itself.

Methods: Observational study of patients participating in a clinical study on transfusion alternatives. From 11 patients, whole blood samples before and 24 hours after surgery were collected for GE array analysis of 114 cytokines, followed by reverse transcriptase RealTime-PCR for selected genes in 56 patients, of which 19 received washed and 13 received unwashed autologous blood and 24 were control patients.

Results: After surgery, IL-8, TNFsf10 and TNFsf13B showed most frequent up- and down-regulations and were selected for PCR analysis. Overall, post-surgical inter-patient variations were large and exceeded the differences observed with or without autologous blood reinfusion. Unwashed salvaged blood re-infusion was associated with slightly more up-regulation of TNFsf13B GE compared to controls or patients who received washed salvaged blood.

Conclusion: Both autologous washed or unwashed salvaged blood re-infusion after hip surgery was associated with minor systemic changes in cytokine GE. The results suggest a more pro-inflammatory response after unwashed as compared to washed salvaged blood.

Clinical relevance: Salvaged blood re-infusion adds minor immuno-modulatory alterations compared to the hip replacement surgery itself. Unwashed blood may be somewhat more pro-inflammatory.

Allogeneic blood transfusions (ABT) in orthopaedic surgery have been associated with an increased risk for post-operative infections [7]. In order to diminish the use of ABT, various blood saving strategies are employed. In particular in orthopaedic surgery, post operative re-infusion of salvaged blood is an often used strategy [12,20,31]. Preliminary studies found a decrease of post-operative infections using salvaged blood as compared to patients transfused with allogeneic blood [31]. This protection against postoperative infections has been attributed to the stimulation of natural killer (NK) precursor cells by re-infusion of salvaged blood in contrast to the suppression of NK cells, which may occur after joint surgery and ABT [18].

Salvaged blood contains cytokines and activated leukocytes (neutrophils and macrophages), which are attracted by the local exudate at the surgical site. As compared to peripheral blood values prior to surgery, several pro- and anti-inflammatory cytokine genes become upregulated in neutrophils present in salvaged blood during the maximally 6 hours allowed between collection and re-infusion [2,5,6,11,13,19,21,28]. Before re-infusion, the collected salvaged blood can be washed to eliminate these cytokines, or filtered to remove debris and (activated) leukocytes, but not cytokines [1,2]. Filtration however, may activate the complement system [13,19], while washing of shed blood was reported to increase tumor necrosis factor-α (TNF-α) [6].

Re-infusion of filtered or washed salvaged blood results in a, mostly transient, increase of some cytokine levels in vivo (e.g. IL-6), with slight differences between the two modalities [1,5,18,21,28,32].

Additionally, after re-infusion of salvaged blood transient elevations in plasma cytokine levels have been measured, which may be associated with fever. Approximately 30% of patients [28] experience mild febrile transfusion reactions after re-infusion, although after orthopaedic surgery without re-infusion of salvaged blood a rise in temperature has also been observed [14]. It is however not elucidated whether these passively administered factors have an effect on the host immune reactivity. We investigated a possible effect on the recipient immune status 24 hours after hip surgery and autologous salvaged blood re-infusion. We compared whether re-infusion of salvaged blood had resulted in different cytokine gene expression profiles compared to controls and secondly whether washed or unwashed salvaged blood re-infusion resulted in different cytokine gene expression patterns.

MATERIAL AND METHODS

An observational study in elective orthopaedic hip replacement (THR) surgery patients, selected from a randomised controlled trial (RCT; Current Controlled Trials number, ISRCTN 96327523; Netherlands Trial Number, NTR303) evaluating various blood management

strategies in orthopaedic surgery. In this study, patients were randomised for either no autologous re-infusion (control group), or an intra- and post-operative blood salvage system that washes and concentrates the collected red blood cells before return to the patient (OrthoPAT®, Haemonetics, Breda, Netherlands), or a post-operative re-infusion system that filters unwashed salvaged blood (DONOR™ system, Van Straten Medical, Nieuwegein, the Netherlands). Both devices are FDA-approved. The ethics committee approved the protocol and the amendments, and all patients provided written informed consent before enrolment. Details of the study and overall results are reported separately [29].

From September 2006 until February 2008 pre and post operative whole blood samples from 56 patients undergoing elective THR surgery at the Leiden University Medical Center, Leiden, The Netherlands, were collected for RNA analysis. Exclusion criteria were revision surgery, rheumatoid arthritis and erythropoietin use. Initially, a pilot study of 11 patients was performed in order to select genes for additional reverse transcriptase RealTime-PCR (RT-PCR), which was based on gene expression changes after surgery. IL-8 data of the pilot study were used to calculate the necessary sample sizes (power of 90 and alpha of 0.05) to detect a difference in post-operative gene expression between cell saver recipients (washed salvaged blood) and controls (hypothesis 1), between unwashed drain reinfusion patients and controls (hypothesis 2) and between cell saver (washed) and unwashed drain reinfusion patients (hypothesis 3). These comparisons resulted in group sizes of 22 control patients, 58 unwashed wound drain reinfusion recipients and 10 cell saver recipients.

Some samples were invalid for analysis, leaving a total group of 24 control patients (i.e. with a regular wound drain), 13 drain re-infusion patients, and 19 cell saver patients. Thus the sample size was adequate to evaluate hypothesis 1, but sample sizes were inadequate for hypotheses 2 and 3.

Patient whole blood samples were taken before and 24 hours after surgery. Two samples of 500 mL were stabilised as soon as possible with RNALater (Applied Biosystems/Ambion, Austin, TX USA) and stored at -40˚C. RNA extraction was performed using the RiboPure™-blood kit (Applied Biosystems/Ambion, Austin, TX USA) according to standard protocol including the optional DNase treatment. RNA quantity was determined with the NanoDrop 1000 photospectrometer (NanoDrop products, Wilmington, DE, USA). Additionally, good RNA integrity was verified for a random selection of samples with the Bioanalyzer (Agilent Technologies, Santa Clara, CA USA). Preceding experiments had shown that the quality and quantity of isolated RNA are not influenced by storage time up to 72 hours at 2-6˚C before RNA stabilisation (data not shown).

Gene expression analysis was performed with the commercially available cytokine array (OHS-021, SA Biosciences™, Frederick, MD USA). This array contains 114 hybridisation spots for cytokines. Amplification and labelling of RNA was performed according to manufacturer's protocol of the TrueAMP 2.0 kit (SA Biosciences™, Frederick, MD USA). The cRNA was hybridised for 18 hours upon the array. The spots on the arrays were visualized

with a ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA USA). Quantification of spot intensities was conducted using the GEArray Analysis Suite (SA Biosciences™, Frederick MD USA). Spot intensities were corrected for inter-array variation by subtracting the average value of spot intensities from all individual values per array. Gene expression values were expressed as ratios that compared the post surgery sample to the pre surgery sample.

The two-step reverse transcriptase Real-Time PCR assays were developed and executed as described earlier [27]. All assays had a taqman® probe with FAM dye and a minor groove binding non-fluorescent quencher (Applied Biosystems Inc., Foster City, CA USA).

For all patient samples, cDNA was synthesized from the extracted RNA with the SuperScript® II Reverse Transcriptase, using random primers (Invitrogen Corporation, Carlsbad, CA USA). The 7500 Fast RT-PCR instrument (Applied Biosystems Inc., Foster City, CA USA) was used on standard modus and standard program with the Gene Expression master mix (Applied Biosystems Inc., Foster City, CA USA). Each sample was run in quadruplo. A measurement was considered an outlier if it had a value which deviated more than 0.4 threshold cycle (Ct) from the average of the data points and was then discarded. If a second outlier was detected, the entire sample was discarded, and repeated in a new run. In each run a reference sample, consisting of pooled cDNA of 6 healthy adult blood donors, was taken along in 4-fold. All RT-PCR output was corrected for the reference sample from the same run. Relative quantification (RQ) was calculated as post/pre operative gene expression ratio within each patient.

All data were analysed statistically with SPSS version 15.0 for windows (SPSS Inc., Chicago, IL USA). The fold change in gene expression after surgery was compared between the control, cell saver and drain groups using Mann-Whitney U tests.

Changes in RT-PCR gene expression were correlated with clinical variables, including volume of re-infusion and infection rate. For this purpose, Spearman correlations were performed. We considered a p-value <0.05 statistically significant. Data are expressed as medians with interquartile ranges (IQR).

RESULTS

Patient characteristics showed no differences between the three study groups (24 control patients, 19 washed cell saver and 13 unwashed drain patients), except for a larger amount of blood loss in the drain group compared to the other groups (Table 1). Although cell saver recipients received smaller volumes of re-infused blood than those of unwashed drain blood, the amount of re-infused RBCs was comparable because of the more concentrated haemoglobin levels in the washed cell saver blood compared to unwashed salvaged blood.

Table 1. Patient demographics

	Control (N=24)	Washed salvaged blood (N=19)	Unwashed salvaged blood (N=13)
Age, median (IQR), years	65 (50-75)	70 (67-78)	64 (51-73)
Gender, N, Male	10	6	7
Weight, median (IQR), kg	76 (68-84)	73 (63-82)	78 (73-90)
Corticosteroids, N	1	1	0
NSAIDs, N	5	5	3
CRP, median (IQR), mg/L	3 (3-6)	3 (3-8)	5 (3-8)
BSE, median (IQR), mm/h	10 (6-30)	8 (5-29)	9 (5-14)
Surgery			
Duration of surgery, median (IQR), minutes	120 (105-150)	120 (105-133)	135 (110-150)
Type prothesis, cemented	11	15	7
Total blood loss during surgery, median (IQR), mL	443 (305-673)	235 (120-400)	370 (175-725)
Post-surgery			
Total blood loss post surgery, median (IQR), mL	270 (235-450)	265 (155-630)	780 (460-910)
autologous re-infusion, median (IQR), mL	N.A.	115 (70-200)	340 (200-500)
Hemoglobin, day +1 median (IQR), mmol/L	6.4 (6.0-6.8)	6.5 (6.1-7.0)	6.3 (5.8-7.7)
Leukocytes, day+1 median (IQR), 10^9/L	8.2 (7.4-9.2)	7.9 (6.8-9.2)	9.2 (8.4-10.4)
Temperature, day +1, median (IQR), °C	38.0 (37.7-38.2)	37.9 (37.5-38.0)	37.8 (37.1-38-5)
Hospital stay, median (IQR), days	7 (6-9)	8 (7-10)	7 (6-10)
Complications up to day 14			
Infections, N	0	1	0
Wound leakage, N	2	2	1
Other complications, N[a]	4	2	0

None of the patients has had previous CVA. None of the patients received antifibrinolytic agents. NSAIDs were stopped 3-10 days prior to surgery. None of the patients died. N.A. not applicable.
[a] Two control and two cell saver patients had cardio-vascular events. One hip dislocation and one pulmonary embolism occurred in the control group.

In the pilot group of 11 patients, 44 out of 114 cytokine genes were expressed above the detection limit on at least one of the arrays (Figure 1A). Seventeen of these showed at least a 2-fold change after surgery in minimally one patient (Figure1B; Table 2) of which nine genes were up-regulated, 3 showed down-regulations and 5 genes exhibited up-regulations in some, and down-regulations in other patients. The most striking responses were seen in the pro-inflammatory cytokines IL-8, TNF superfamily 13B (TNF sf13B) and inTNF sf10: IL-8 showed up-regulation in 3, and was down-regulated in 3 other patients. Control patients showed only minor changes in IL-8 gene expression, while in patients receiving unwashed

salvaged blood the gene was up- and in most patients receiving washed salvaged blood down-regulated. TNF super family 13B (TNFsf13B) was more than 2-fold up-regulated after surgery in 5 of 11 patients, and TNFsf10 was up-regulated in 3 and down-regulated in 1 patient. The remaining 14 genes showed only changes in a single patient (n=10) or showed a mixed response (up- and down-regulation in different patients) and were not further addressed. RT-PCR in the pilot patients confirmed the array results and IL-8, TNFsf10 and TNFsf13B were subsequently studied in extended patient groups by RT-PCR.

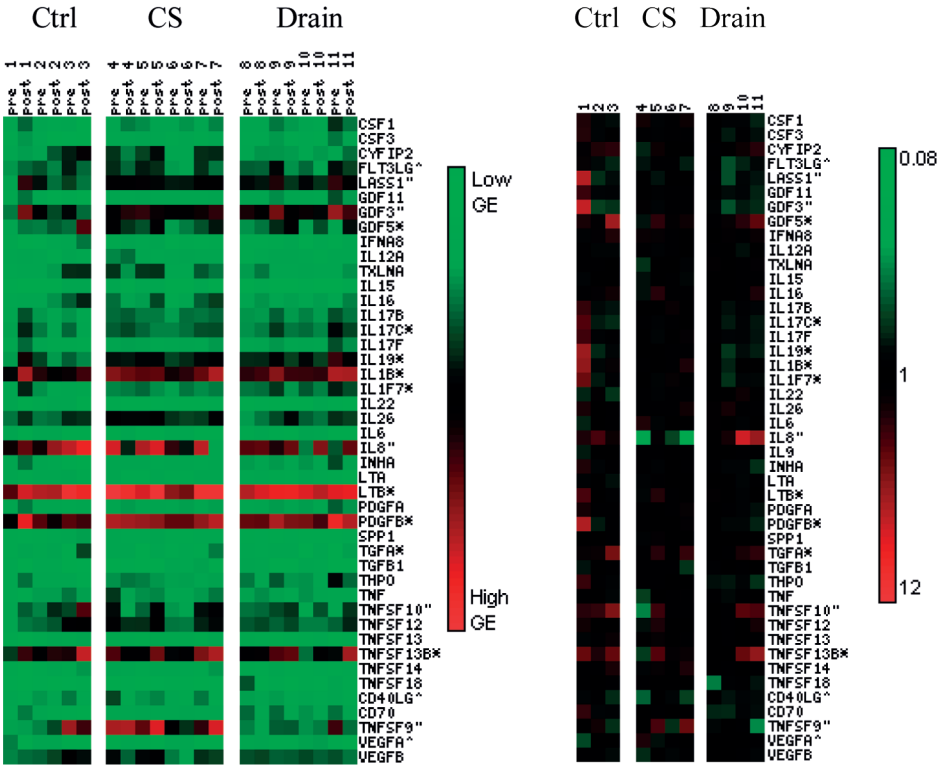

Figure 1.

Ctrl=control group; CS=washed salvaged blood by cell saver; Drain=unwashed salvaged blood by drain reinfusion.

A: Gene expression (GE) of all genes detectable on at least one array. Red presents high GE, green is low GE.

B: ΔGE of all detectable genes. Both post-RBC samples are compared to the pre sample of each patient. Green is a down-regulation, black indicates no major changes and red represents up-regulation.

* Genes which exhibit at least a 2-fold up-regulation after surgery in at least one patient.

^ Genes exhibiting at least a 2-fold down-regulation after surgery in at least 1 patient.

"Genes exhibiting at least 2-fold up-regulation and a 2-fold down-regulation after surgery in at least one patient each.

Figures were constructed with matrix2png 1.0.7 [24].

Table 2. Gene expression (GE) fold changes after surgery of the pilot group on the arrays

	Control			Washed salvaged blood				Unwashed salvaged blood			
CD40-L	0.81	0.87	0.60	**0.39**	1.1	0.78	0.51	0.70	0.98	0.81	0.74
FLT3-L	1.3	0.75	0.64	0.70	0.92	0.87	0.65	**0.45**	0.97	0.64	0.76
GDF-3	**5.3**	**0.50**	0.57	1.2	0.81	1.2	1.2	**0.44**	0.79	0.67	0.61
GDF-5	1.5	1.2	**3.9**	0.70	1.7	1.2	1.4	1.3	1.2	1.6	**2.4**
IL-1β	**3.6**	0.75	1.3	0.87	1.0	1.1	1.5	0.65	1.1	0.96	0.95
IL1-F7	**2.7**	0.67	0.86	1.1	0.80	1.3	1.1	0.57	1.0	0.78	0.76
IL-8	1.6	**2.0**	1.4	**0.19**	1.3	**0.50**	**0.08**	1.5	1.1	**5.7**	**3.7**
IL-17C	**2.2**	0.67	0.61	1.1	0.84	1.1	1.0	0.68	1.1	0.85	0.69
IL-19	**3.8**	0.61	0.77	0.98	0.99	1.2	1.1	0.55	1.0	0.73	0.65
LASS1	**4.7**	0.58	0.73	0.96	0.70	1.0	1.0	0.45	0.93	0.77	0.70
LT-β	**2.0**	0.95	0.85	0.78	1.6	0.87	1.0	1.0	1.2	0.82	1.1
PDGF-β	**4.4**	0.67	0.87	0.91	0.95	1.0	1.3	0.77	0.98	0.76	0.65
TGF-α	1.3	1.3	**2.9**	0.88	1.6	1.1	1.8	1.1	0.97	1.5	1.7
TNFsf9	1.0	1.4	0.58	1.1	**2.1**	0.59	2.6	1.0	0.76	1.1	**0.29**
TNFsf10	1.6	1.7	**3.1**	**0.30**	1.9	0.89	1.1	1.2	1.1	**2.3**	**2.1**
TNFsf13B	**2.6**	1.5	**2.5**	0.53	**2.2**	1.3	1.3	1.1	1.0	**2.6**	**3.5**
VEGF- α	**0.48**	1.0	0.97	1.5	0.72	0.92	0.97	0.98	1.1	1.0	0.88

All GE values are ratios of GE after surgery with or without autologous re-infusion in comparison with GE before surgery. Each column represents a patient. More than two-fold changes are presented in bold.

In the extended population, however, IL-8 gene expression after surgery showed large inter-individual differences of more than 2 logs, mostly down-regulations (Table 3; Figure 2), in all three groups of control patients, cell saver and drain recipients. In most patients TNFsf10 and TNFsf13B gene expression levels were elevated after surgery. These increases were higher in the patients receiving unwashed salvaged blood than in control patients and in patients receiving washed salvaged blood, which was significant for TNFsf13B gene expression between drain and cell saver patients (Table 3; Figure 2).

None of the relevant clinical variables clearly correlated with gene expression of IL-8, TNFsf10 and TNFsf13B (Table 4), including volume drainage blood re-infused, neither within nor between the washed cell saver and unwashed drain groups.

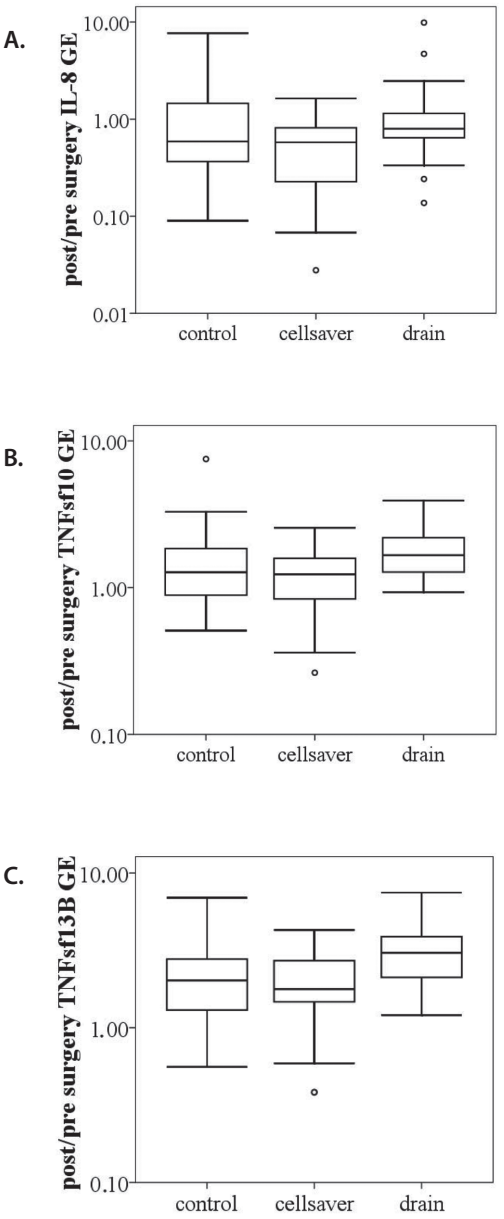

Figure 2. Boxplots showing median fold changes in gene expression (GE) measured with RT-PCR. A: IL-8, B: TNFsf10, C: TNFsf13B.

Table 3. Fold changes in gene expression (GE) for 3 selected cytokines with RT-PCR in the total group

	Control	washed salvaged blood	unwashed salvaged blood	Ctrl vs CS p-value	Ctrl vs drain p-value	CS vs drain p-value
N	24	19	13			
Post/pre IL8 median (IQR)	0.59 (0.36-1.62)	0.57 (0.16-0.85)	0.79 (0.48-1.80)	0.3	0.4	0.081
Post/pre TNFsf10 median (IQR)	1.27 (0.86-1.87)	1.23 (0.81-1.60)	1.66 (1.12-2.42)	0.8	0.086	0.081
Post/pre TNFsf13B median (IQR)	2.02 (1.28-2.83)	1.78 (1.44-2.75)	3.05 (1.75-4.22)	0.6	0.14	0.033

All GE values are ratios of GE after surgery with or without autologous re-infusion in comparison with GE before surgery. CS: cell saver etc.

Table 4. Associations of gene expression (GE) with clinical variables

		IL-8		TNFsf10		TNFsf13B	
	N	correlation coefficient	p-value	correlation coefficient	p-value	Correlation coefficient	p-value
Age	56	-0.238	0.078	-0.121	0.4	-0.052	0.7
Gender	56	-0.221	0.10	-0.228	0.091	-0.21	0.12
before surgery							
NSAIDs	56	-0.132	0.3	-0.198	0.14	-0.035	0.8
during surgery							
Duration of surgery	52	0.225	0.11	-0.115	0.4	-0.179	0.2
Blood loss during surgery	56	0.062	0.6	0.003	1.0	0.104	0.4
after surgery							
Blood loss after surgery	51	0.212	0.13	0.044	0.8	0.054	0.7
Reinfusion volume	56	0.056	0.7	0.149	0. 3	0.190	0.16
Hemoglobin	56	0.141	0.3	-0.052	0.7	-0.065	0.6
Leukocytes	55	-0.012	0.9	0.273	0.044	0.272	0.045
Temperature	48	0.018	0.9	0.272	0.064	0.345	0.018

Spearman correlations were performed.

DISCUSSION

Autologous salvaged blood re-infusion is a widely used alternative for allogeneic blood transfusions and preliminary reports claim a reduced incidence of post-operative infections through beneficial immunomodulation. Although salvaged blood contains cytokines and activated cells which are re-infused into the patient, an effect on the recipient immune system is however less documented. We investigated alterations in patient cytokine gene expression profiles in relation to autologous salvaged blood product re-infusion and secondly whether washing of salvaged blood made a difference. For initial selection of relevant cytokines we performed a pilot study investigating 114 cytokine genes on expression array in 11 patients; only 3 cytokines came up as candidate altered gene expressions. These were subsequently evaluated by PCR in an extended cohort of 56 patients.

Our study had some limitations. Despite homogeneity of the population, we still detected major inter patient variation in gene expression, for which we were unable to find a possible explanation. Also, not enough patients were available to comply with the sample sizes required to detect statistically significant differences in gene expression ratio's between control and drain patients. However, as gene expression ratio's, analysed by PCR, were smaller than observed on the array results, not the sample size, but the smaller differences in gene expression ratio's explain the non-significant results. As only one of the control patients had received allogeneic RBC transfusions during or within 24 hours after sampling, we were not able to compare the effect of autologous re-infusion with the effect of allogeneic RBCs on cytokine gene expression. By only measuring before and 24 hours after surgery, we might have missed a peak in gene expression [3,4,16], although a previous study that investigated the surgery effect on cytokine gene expression showed changes only after 24 hours, and not at 6 hours after surgery [21].

We found in the pilot study patients that IL-8, TNFsf10 and TNFsf13B showed most frequently up-or down-regulations. However in the larger patient population, increases of IL-8, TNFsf10 and -13B gene expression after surgery were less pronounced (Table 3). Surgery by itself had major effects on the gene expression profiles and additional effects of salvaged blood re-infusion were minor, although small additional up-regulation remained present after re-infused unwashed drain blood.

Despite re-infusion of inflammatory cytokines in drain blood and leukocytes with up-regulated cytokine genes in cell saver blood, the re-infusion of this salvaged blood showed, after 24 hours, only minor systemic changes in cytokine gene expression in excess of the surgical procedure itself. Although these minor up-regulations of IL-8, TNFsf10 and TNFsf13B involve genes which are among other effects, all associated with NK cell activation [10,15,18,30], the most potent NK-cell activating cytokines (IL-2, -12, -15, -18 and interferon-α) did not show changes on the array, although their activation may have occurred before or after the time point of 24 hours post-surgery [33].

The main function of IL-8 is neutrophil chemoattraction, but it also stimulates neutrophil and T-cell activity [23]. Control patients did not show changes in IL-8 gene expression, while patients receiving unwashed salvaged blood often showed up-regulation and patients receiving washed salvaged blood mostly showed down-regulation of this gene. TNFsf10 encodes for TNF-related apoptosis inducing ligand (TRAIL), has a function in apoptosis induction and plays a role linking the adaptive and innate immune system [25], TNFsf13B is associated with proliferation of B-cells [6], also referred to as B-cell activating factor (BAFF) [26]. The BAFF-Receptor induces cell signalling through the IKKβ and NF-κB pathways, promoting transcription of IL-8, TNFsf13B and CD40L [17].

The large inter-patient variation in gene expression, was not explained by (type of) salvaged blood transfusion, or associated to other clinical variables including postoperative anaemia, leukocytosis, fever, blood loss and volume of re-infused salvaged blood. Absence of an association between IL-8 gene expression and re-infused volume, has been previously published [9]. Rather, other inter-individual (genetic) or unknown environmental factors may play a role.

We found only one other study that evaluated cytokine gene expression in neutrophils after hip arthroplasty [11]. This study compared the neutrophils in salvaged blood to those in pre-surgical and post-surgical circulating blood in the same patient without re-infusion of the salvaged blood. The authors found that other cytokines, such as interleukin-1 receptor antagonist (IL1RA), interleukin-18 receptor 1 (IL18R1), macrophage migration inhibitory factor (MIF), and macrophage inflammatory protein 3alpha (CCL20) were upregulated both in the patient and in salvaged blood, whereas interleukin-8 receptor beta (IL8RB/CXCR2) was consistently downregulated. Our findings, reflecting the total leukocyte population, did not only report systemic gene expression changes after surgery, but also compared gene expression changes after re-infusion of washed or unwashed salvaged blood with the presurgical situation and control surgery patients. We found a stronger up-regulation of TNFsf13B after receiving autologous unwashed blood compared to controls or washed salvaged blood. The differences in complications can not be related to either the washed or unwashed re-infusion of blood, since it is a post-hoc analysis with inadequate power for detecting a relevant difference between the groups.

In conclusion, inter-patient variations of unidentified cause showed more changes in cytokine gene expression after hip surgery than re-infusion of shed salvaged blood. Unwashed filtered drain blood showed more up-regulation of the pro-inflammatory cytokines IL-8, TNFsf10 and -13B compared to washed salvaged blood or controls.

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