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Original article

Capsular inflammation after immediate breast reconstruction – gene expression patterns and inflammatory cell infiltration in irradiated and non-irradiated breasts

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Abstract

Background
Capsular contracture following post-mastectomy radiotherapy (PMRT) is commonly seen in patients with implant-based immediate breast reconstruction (IBR). Further understanding of the underlying biology is needed for the development of preventive or therapeutic strategies. Therefore, we conducted a comparative study of gene expression patterns in capsular tissue from breast cancer patients who had received versus those who had not received PMRT after implant-based IBR.

Methods
Biopsies from irradiated and healthy non-irradiated capsular tissue were harvested during implant exchange following IBR. Biopsies from irradiated (n=13) and non-irradiated (n=12) capsules were compared using Affymetrix microarrays, to identify the most differentially regulated genes. Further analysis with immunohistochemistry was conducted in a subset of the material to compare the presence of T cells, B cells, and macrophages.

Results
Enrichment testing with Gene Ontology (GO) analysis revealed that the 227 most differentially expressed genes were mainly involved in an inflammatory response. Twenty-one GO biological processes were identified (p<0.05, FDR<5%), several with B cell-associated inflammation. CIBERSORT analysis identified macrophages as the most common inflammatory cell type in both groups, further supported by immunostaining of CD68. Strikingly, radiation increased B-cell infiltration in the capsular region of biopsies, as quantified by immunostaining of CD20 (p=0.016).

Conclusions
Transcript analysis and immunohistochemistry revealed inflammatory responses in capsular biopsies regardless of radiotherapy. However, the radiation response specifically involved B cell-associated inflammatory responses.

Keywords
Implant-based breast reconstruction; radiotherapy; capsular contracture; inflammation
1. Introduction

Radiotherapy (RT) is an important part of adjuvant treatment in breast cancer since it significantly reduces the risk of local recurrence and death (1). It does, however, also affect surrounding tissue such as underlying muscle and subcutaneous fat, which has detrimental consequences in breast reconstruction. The effects of RT involve chronic inflammatory changes, tissue remodelling, and fibrosis (2). The tissue response can be substantial, resulting in capsular contracture around the implant, impaired cosmetic outcome, psychological distress, and pain (3). In severe cases, the implant may have to be removed and replaced with a new implant after capsular revision, or the breast may need to be re-reconstructed with autologous tissue. A Swedish study has estimated the 5 year failure rate in breast cancer patients with implant-based reconstruction to be 28.2 % for previously, and 25.2 % for postoperatively irradiated patients ($p < 0.001$) (4). The aetiology of capsular contracture is multifactorial and additional factors, such as placement of the implant (submuscular versus prepectoral), implant surface texture, bacterial colonization and seroma/hematoma, may affect the outcome (5). Potential explanations of the underlying aetiology of radiation-induced fibrosis as a consequence of, for example, genetic variation are as yet inconclusive (6). We have previously described variation in gene expression patterns both in irradiated recipient vessels from autologous flap reconstructions (7) and in adipose tissue in the irradiated breast (8), but to our knowledge, no study has described gene expression patterns in irradiated implant capsules.

Ameliorating capsular inflammation to reduce capsular contracture has been proposed in an aesthetic setting of breast augmentation (9). Studies after aesthetic breast augmentation have shown that in the absence of irradiation, capsular contracture is associated with inflammatory cell recruitment together with an increased expression of toll-like receptor 4 (TLR4) (10) and cysteine leukotriene receptor 2 (CysLTR-2) (11) in fibroblasts within the capsular tissue.
Leukotriene inhibitors have been suggested as a prophylactic treatment to reduce the risk of capsular contracture in aesthetic surgery (12). Interleukin 8 (IL8) and metallopeptidase 4 (TIMP4) have been suggested as potential key diagnostic and prognostic biomarkers (13). However, most previous studies have been restricted to aesthetic breast augmentation as opposed to breast cancer reconstruction, where radiotherapy may further enhance inflammatory cell recruitment and fibrosis formation. Lipa et al. showed that the Wnt signalling pathway, previously shown to be involved in the pathogenesis of radiation-induced fibro-proliferation, may play an important role in capsular contracture after expander breast reconstruction (14). However, that study was limited to only 3-5 patients and did not evaluate gene expression patterns.

Given the limitations of the present data, we took an unbiased approach to evaluate gene expression patterns in irradiated and non-irradiated breasts in the search for future therapeutic targets. The latter could be of paramount importance for reducing the risk of developing radiotherapy-induced capsular contracture in the increasing number of breast cancer patients that undergo immediate breast reconstruction (IBR).

2. Material and methods

2.1 Human Tissue Specimens. Forty patients undergoing implant exchange in 44 breasts after previous implant-based IBR were included after informed consent. Patients who had a non-standard irradiation dose or who had recently undergone implant exchange for other reasons were excluded. Capsular biopsies of irradiated (n=23) and healthy non-irradiated (n=23) tissues were harvested during implant exchange at two breast centres. A full-thickness 10x10mm capsular biopsy was harvested from the lateral lower quadrant of the breast. Biopsies were divided into two parts and stored in Allprotect Tissue Reagent® (Qiagen,
Hilden, Germany) and stored at –80 °C for RNA purification, and in formalin for immunohistochemistry. No samples were pooled. All samples were registered at the Stockholm Medical Biobank (nr 914). The study was approved by the regional ethical review board at Karolinska Institutet in Stockholm (2017/1504-31/2) and was performed in agreement with institutional guidelines and the principles of the Declaration of Helsinki.

2.2 RNA extraction. The RNeasy Lipid Tissue kit® (Qiagen) was used according to the manufacturer’s protocol to extract RNA. The quality of the RNA was assessed by microcapillary electrophoresis using an Agilent Bioanalyzer® with RNA 6000 Pico Kit and Agilent 2200 TapeStation with RNA ScreenTape (Agilent, Santa Clara, California, USA). The quantity of RNA was measured by ultraviolet spectrophotometry with a NanoDrop® ND-1000 UV – Vis Spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). Whole-transcriptome expression analysis was performed using the GeneChip® WT Pico Reagent Kit (Affymetrix, Santa Clara, California, USA) by processing each sample of RNA from the total RNA (50ng). This kit produces amplified and biotinylated sense-strand DNA targets for hybridization to Clariom D (human) arrays.

2.3 Gene expression profiling. Microarray analysis was performed by the core facility for Bioinformatics and Expression Analysis at Karolinska Institutet. Affymetrix® Clariom D human oligonucleotide microarrays (Affymetrix, Santa Clara, California, USA) were used for gene expression profiling. CEL files from scanning were processed in the Transcriptome Analysis Console (TAC) using the SST-RMA method. A gene list was created by extracting transcripts registered as encoded genes according to The National Center for Biotechnology Information (NCBI)’s database for gene-specific information, Entrez Gene, http://www.ncbi.nlm.nih.gov/gene, and present in at least one of the groups. Irradiated biopsies (RT+) were compared to non-irradiated biopsies (RT-) to extract the genes that were
most differentially expressed. Enrichment analysis of differentially expressed genes was conducted by using The Molecular Signatures Database (MSigDB), https://www.gsea-msigdb.org/gsea/msigdb/index.jsp, to identify different levels of gene sets and pathways associated with the radiotherapy responsive genes.

2.4 Estimating the composition of immune cells. To estimate the immune cell composition in the sample based on the RNA expression pattern and to quantify the relative levels of distinct immune cell types within a complex gene expression mixture, the analytical platform CIBERSORT (https://cibersort.stanford.edu/) was used. Different cell types were also compared in the RT- and RT+ groups using immunohistochemistry.

2.5 Immunohistochemistry. The formalin-fixed tissues were histo-processed in an automated tissue processing machine (VIP 3000, Miles Scientific) and embedded in paraffin. 4-μm-thick FFPE (formalin-fixed, paraffin-embedded) sections were mounted on glass slides (Superfrost+, Thermo Scientific) and heated for 3 hours at 56°C. After deparaffinization in xylene and rehydration in alcohol, heat-induced epitope retrieval was performed using a Decloaking Chamber (Biocare Medical) set for 5 min at 110°C in Citrate buffer pH 6 (Sigma C-9999). For quenching of endogenous peroxidase, a 30-minute incubation in 0.15 % hydrogen peroxidase was performed at room temperature, followed by a 30-minute blocking step using 1% bovine serum albumin (BSA). Primary antibodies were diluted 1/2000 (CD3), 1/600 (CD20), 1/1000 (CD68), in 1% BSA, incubation at +4°C overnight in a humid chamber. The secondary biotinylated antibody (Vector Laboratories) was diluted 1/200 and incubated for 30 min at room temperature. This was followed by 30 min incubation with avidin-biotin enzyme complex (Vectastain) ELITE ABC kit (HRP, Vector Laboratories). For visualization, the peroxidase substrate DAB was used (ImmPACT DAB SK-4105, Vector Laboratories) for 3 min. The sections were counterstained in Mayer’s hematoxylin for 1 min.
followed by dehydration with graded alcohols, xylene, and coverslipped with Mountex. Eight biopsies (four from each group) were analyzed, using one section per biopsy. Three equally sized high power fields (x20) were selected at random and oriented horizontally to the region of the capsular surface that was most intact, with a depth of 200 µm below the surface. The number of CD3, CD20, and CD68 positive cells were independently counted by two blinded evaluators and the results summarized and presented as a mean value.

**Statistical analysis**

Group analysis was used to test differences between the irradiated and non-irradiated groups. Expression levels in irradiated and non-irradiated sample groups were compared using moderated t-tests as implemented in the BioConductor Limma package. Only genes assessed by TAC to be expressed in at least one of the treatment conditions, to contain protein-coding exons, and to have an entry in Entrez Gene were included in further analysis. Genes with a p-value <0.05 were selected for further enrichment testing. Gene ontology biological functions and Reactome gene sets with a corrected p-value <0.05, false discovery rate (FDR) <5%, were considered significantly overrepresented.

**3. Results**

3.1 Human Tissue Specimens. A total of 44 patients were enrolled, two of whom had bilateral biopsies from each breast. Biopsies from 25 patients met the criteria for RNA quality and quantity and were included in the gene expression analysis (Figure 1). The median (range) time from completion of radiotherapy to capsular biopsy was 41 (18 – 304) months and the median radiation dose was 50 (46 – 50) Gy (Table 1). All implants were submuscular and textured, with Mentor CPG and Becker 35 expanders the most common models. Body mass index (BMI), age at the time of biopsy, implant size, and the number of surgical re-entries
into the implant cavity (e.g. hematomas and abdominal advancement) and non-surgical complications (e.g. hematomas and seromas) were evenly distributed across the irradiated and non-irradiated groups. However, four more patients had previously undergone an implant exchange in the irradiated group, and there were three more patients with a previous infection in the non-irradiated group (Table 1).

3.2 RNA extraction. Biopsies from 23 irradiated and 23 non-irradiated breasts were collected for RNA extraction. RNA from 13 irradiated and 12 non-irradiated biopsies had a RNA integrity number greater than 5 and a yield of more than 100ng, and were selected for gene expression analysis.

3.3 Gene expression. Microarray experiments were conducted at two time points and therefore subjected to a batch-control analysis showing a negligible temporal effect, which further validated the reproducibility of the experiment. Altogether 3422 transcripts, registered as encoded genes according to Entrez Gene, were present in at least one of the groups. A set of the most differentially expressed transcripts, with a raw p-value below 0.05, was selected for enrichment testing (n=227). Gene ontology (GO) analysis showed that the selected radiation responsive genes were mainly involved in inflammatory response among the top 21 identified GO biological processes (p<0.05; FDR<5%). Both innate and adaptive immune responses were represented. The top three GO biological processes identified were humoral immune response mediated by immunoglobulins followed by complement activation, and B cell-mediated immunity (Table 2). In the Reactome gene sets analysis, scavenging of heme from plasma, C2, and C4 activators and binding/uptake of ligands by scavenger receptors were the most dysregulated (Table 3).

2.4 Estimating immune cell composition. We used CIBERSORT to estimate the immune cell composition of the 25 samples (irradiated and non-irradiated combined) and quantify the
relative levels of different cell types in a mixed cell population. As shown in Figure 2, macrophages and T cells were estimated to be the most common immune cells in the biopsy material, although without any significant differences in their numbers between the irradiated and non-irradiated groups. However, there was a significantly greater number of γδT cells (p=0.022), and a tendency towards more naïve B cells (p=0.077) and activated NK cells (p=0.077) in the irradiated group.

3.5 Immunohistochemistry. Cell-specific markers of the most relevant cell types according to enrichment testing were analyzed. Of the 25 biopsies that were included in gene expression analysis, only eight had an intact morphology of the capsular surface and were of adequate staining quality to enable cell quantification. This was mainly as a consequence of surgical constraints, which resulted in uncertainty regarding orientation either during the sampling, or during paraffin embedding when biopsies were small. Cell counts were obtained by two blinded evaluators. When all the stained sections were analysed as a whole, there were significantly more CD68+ cells compared to both CD3+ cells (p=0.0062) and CD20+ cells (p=0.0025), which corresponded to the results obtained using CIBERSORT. Further comparison of the RT+ and RT- groups revealed no differences apart from a greater number of CD20+ cells in the RT+ biopsies (p=0.016) (Figure 3). As seen in Figure 3, B-cell infiltration was restricted to irradiated capsules. Indeed, when bilateral capsular biopsies were obtained and stained for B cells (CD20+), there was an absence of B cells in the non-irradiated capsule, while there was an abundance of CD20+ B cells in the irradiated capsule (Figure 4).

4. Discussion

We undertook an exploratory study on the underlying human biology of breast reconstruction by comparing irradiated and non-irradiated capsular biopsies since IBR is an integral part of
breast cancer treatment. Our results showed the occurrence of inflammatory responses irrespective of radiotherapy, while B cell-associated inflammatory responses appeared more specific for irradiated capsules.

Our gene expression results support previous studies showing a sustained innate and adaptive immune response (15), lasting up to several years after radiotherapy exposure. Both innate and adaptive immune responses in capsular biopsies were confirmed with immunohistochemistry, although without significant differences in cell counts for CD3+ T cells and CD68+ macrophages. Somewhat unexpected was the difference in CD20+ B cell counts between irradiated and non-irradiated biopsies, which was further supported by differentially expressed B-cell-related genes. Humoral immune response mediated by immunoglobulins, followed by complement activation and B cell-mediated immunity were identified as the top three GO biological processes that were most differentially by radiotherapy. However, these results should be interpreted with caution due to the limited sample size and exploratory nature of the study.

Regardless of irradiation status, a significant infiltration of inflammatory cells was seen around implants. It is of note that all the implants used in both groups had Mentor’s Siltex microtextured surface. Therefore, the influence of the surface is negligible regarding the comparison between the groups, but is of general interest regarding the interaction at the interface between the implant and the patient’s tissue. We believe that both the gene expression findings, as well as the morphological evaluations, reveal an interesting interplay between macrophages, T-cells and B-cells at the capsular interface between the implant and the breast. This could be of particular importance since the infiltration of immune cells around breast implants has gained increasing attention in recent years following the World Health
Organization’s recognition of breast implant-associated anaplastic large-cell lymphoma (BIA-ALCL) in 2016. The results of the current study need to be interpreted in the light of a number of limitations. Firstly, gene expression data need to be interpreted with caution due to the limited sample size. A generally low RNA quality was noted, which most likely reflects the challenges of handling sensitive tissues in a clinical setting. Compared to previous studies by our group in which irradiated tissue biopsies were compared to internal non-irradiated controls (7,16), the current study showed differences of smaller magnitude between irradiated and non-irradiated samples. This may be due to decreased statistical power in the group (as opposed to paired) analysis, but is more likely explained by considerable inter-individual differences. Paired, synchronous sampling of irradiated biopsies and non-irradiated internal controls offers the possibility to study the effect of radiation alone, but was unfortunately not possible in this cohort. Another explanation for a comparably weak effect in the irradiated group in the current study may relate to the fact that inflammation as a result of the implant *per se* was considerable in both groups. Further in-depth studies in a larger cohort, preferably with bilateral cases of one irradiated and one non-irradiated breast reconstructions, would be of great interest to elucidate the immune responses caused by radiotherapy.

**Conclusion**

Considering the growing population of breast cancer survivors, it is of paramount importance to understand better the biology underlying radiation-induced capsular contracture. We show here that inflammatory responses in capsular biopsies were present regardless of radiotherapy, while B cell-associated inflammatory responses were specific to irradiated tissue. However, this study should be seen as a pilot study. Larger studies with more homogeneous material are
needed in the future to continue elucidating the underlying biology of radiation-induced capsular contracture.
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**Ethical Approval**

The study was approved by the regional ethical review board at Karolinska Institutet in Stockholm (2017/1504-31/2) and was performed in agreement with institutional guidelines and the principles of the Declaration of Helsinki.

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Anonymized data that support the findings of this study are available on reasonable request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

**Declaration of Competing Interest**

The authors declare no conflict of interest.
References

1. Kurtz J. The curative role of radiotherapy in the treatment of operable breast cancer. Eur J Cancer. 2002 Oct;38(15):1961–74.

2. Stone HB, Coleman CN, Anscher MS, McBride WH. Effects of radiation on normal tissue: Consequences and mechanisms. Lancet Oncol. 2003;4(9):529–36.

3. Joanne Sheehan, Kerry A. Sherman TL and JB. Association of information satisfaction, psychological distress and monitoring coping style with post-decision regret following breast reconstruction. Psychooncology. 2008;17(July 2006):363–72.

4. Eriksson M, Anveden L, Celebioglu F, Dahlberg K, Meldahl I, Lagergren J, et al. Radiotherapy in implant-based immediate breast reconstruction: risk factors, surgical outcomes, and patient-reported outcome measures in a large Swedish multicenter cohort. Breast Cancer Res Treat. 2013 Dec;142(3):591–601.

5. Calobrace MB, Stevens WG, Capizzi PL, Cohen R, Godinez T, Beckstrand M. Risk Factor Analysis for Capsular Contracture: A 10-Year Sientra Study Using Round, Smooth, and Textured Implants for Breast Augmentation. Plast Reconstr Surg. 2018;141(4S Sientra Shaped and Round Cohesive Gel Implants):20S-28S.

6. Wei J, Wang B, Wang H, Meng L, Zhao Q, Li X, et al. Radiation-Induced Normal Tissue Damage: Oxidative Stress and Epigenetic Mechanisms. Oxid Med Cell Longev [Internet]. 2019 Nov 12;2019:3010342. Available from: https://pubmed.ncbi.nlm.nih.gov/31781332

7. Halle M, Christersdottir T, Bäck M. Chronic adventitial inflammation, vasa vasorum expansion, and 5-lipoxygenase up-regulation in irradiated arteries from cancer survivors. FASEB J. 2016;30(11):3845–52.

8. Lindegren A, Schultz I, Sinha I, Cheung L, Khan AA, Tekle M, et al. Autologous fat transplantation alters gene expression patterns related to inflammation and hypoxia in
the irradiated human breast. Br J Surg. 2019;106(5):563–73.

9. Lille S, Jacoby J. The potential benefit of preemptive leukotriene inhibitor treatment to breast augmentation/mastopexy surgery. Plast Reconstr Surg. 2018;142(4):610E-611E.

10. Segreto F, Carotti S, Tosi D, Pendolino AL, Marangi GF, Morini S, et al. Toll-Like Receptor 4 Expression in Human Breast Implant Capsules: Localization and Correlation with Estrogen Receptors. Plast Reconstr Surg. 2016;137(3):792–8.

11. Grella E, Grella R, Siniscalco D, Fuccio C, Rossi F, De Novellis V, et al. Modification of cysteinyl leukotriene receptors expression in capsular contracture: Follow-up study and definitive results. Ann Plast Surg. 2009;63(2):206–8.

12. Graf R, Ascenço ASK, Freitas RDS, Balbinot P, Peressutti C, Costa DFB, et al. Prevention of capsular contracture using leukotriene antagonists. Plast Reconstr Surg. 2015;136(5):592e-596e.

13. Kyle DJT, Harvey AG, Shih B, Tan KT, Chaudhry IH, Bayat A. Identification of molecular phenotypic descriptors of breast capsular contracture formation using informatics analysis of the whole genome transcriptome. Wound Repair Regen. 2013;21(5):762–9.

14. Lipa JE, Qiu W, Huang N, Alman BA, Pang CY. Pathogenesis of radiation-induced capsular contracture in tissue expander and implant breast reconstruction. Plast Reconstr Surg. 2010;125(2):437–45.

15. Halle M, Gabrielsen A, Paulsson-Berne G, Gahm C, Agardh HE, Farnebo F, et al. Sustained Inflammation Due to Nuclear Factor-Kappa B Activation in Irradiated Human Arteries. J Am Coll Cardiol [Internet]. 2010;55(12):1227–36. Available from: http://dx.doi.org/10.1016/j.jacc.2009.10.047

16. Christersdottir T, Pirault J, Gisterå A, Bergman O, Gallina AL, Baumgartner R, et al. Prevention of radiotherapy-induced arterial inflammation by interleukin-1 blockade.
Eur Heart J. 2019;40(30):2495–503.
Figure 1. Flow diagram of the patient cohort. Flow chart for the final cohort of biopsies included in the gene expression analysis; 13 irradiated- and 12 nonirradiated biopsies and the immunohistochemistry analysis; 4 irradiated- and 4 non-irradiated biopsies. *RIN, RNA integrity number, is an algorithm for assigning integrity values to RNA measurements.
Figure 2. CIBERSORT analysis of gene expression profiles showed that the ratio of macrophages and T-cells were the most common celltypes. There were no significant differences between the RT- and RT+ groups, except for γδT cells (p=0.022).
Figure 3. Cells were counted in three equally sized high-power fields (x20) randomly oriented along the capsular surface to a depth of 200 µm below the surface. Four irradiated and four non-irradiated biopsies were analyzed. Staining of capsular biopsies for T cells (CD3+), B cells (CD20+) and macrophages (CD68+) revealed that CD68+ cells were significantly more prevalent than CD3+ and CD20+ cells (p=0.0062 and 0.0025 respectively, for RT- and RT+ groups combined). Comparison of staining in irradiated and non-irradiated
groups identified differences for CD20+ cells, with significantly higher frequency in the irradiated material ($p=0.016$). Indeed, B cell infiltration was only present in irradiated biopsies.
Figure 4. Staining of capsular biopsies for B cells (CD20+) in a patient from whom bilateral capsular biopsies were obtained. The non-irradiated right capsule displays an absence of B cells, whereas the irradiated left capsule reveals an abundance of CD20+ B cells.
Table 1. Pre- and postoperative information per breast (n=25)

|                              | Irradiated (n=13) | Non-irradiated (n=12) | P-value |
|------------------------------|-------------------|-----------------------|---------|
| Textured implants            |                   |                       |         |
| Yes                          | 13 (100)          | 12 (100)              |         |
| No                           | 0                 | 0                     |         |
| Previous infection           |                   |                       | < 0.001 |
| Yes                          | 1 (7.7)           | 4 (33.3)              |         |
| No                           | 12 (92.3)         | 8 (66.7)              |         |
| Number of previous implant exchanges | 7 (53.8) | 10 (83.3) | 0.114 |
| ≥1                           | 6 (46.2)          | 2 (16.7)              |         |
| Previous surgical complications^ | 1 (7.7)  | 1 (8.3)               | < 0.001 |
| Yes                          | 12 (92.3)         | 11 (91.7)             |         |
| No                           |                   |                       |         |
| Previous non-surgical complications ^^ | 1 (7.7) | 2 (16.7) | < 0.001 |
| Yes                          | 12 (92.3)         | 10 (83.3)             |         |
| Irradiation dose in Gy/total fractions* | 50/25 (46/23 – 50/25) | N.A. |         |
| Time in months from irradiation to biopsy* | 41 (18 – 304) | N.A. |         |
| Body Mass Index *            | 25 (20 – 30)      | 25 (19/28)            | 0.316   |
| Age at biopsy *              | 52 (33-67)        | 51 (32 – 65)          | 0.479   |
| Implant size in cc*          | 400 (120 – 500)   | 420 (195 – 565)       | 0.392   |

Values in parentheses are percentages unless indicated otherwise. * Median (range) ^ Surgical complication was defined as postsurgical intervention entering the implant cavity ^^ Non-surgical complication was defined as seromas/hematomas.
Table 2. Biological pathways showing the most significant alterations in gene expression (GO biological processes)

| Sets                                                                 | P-value  | FDR corrected P  |
|----------------------------------------------------------------------|----------|-----------------|
| Humoral immune response mediated by circulating immunoglobulin        | 5.27e-13 | 3.87e-09        |
| Complement activation                                                 | 5.53e-12 | 2.03e-08        |
| B-cell mediated immunity                                              | 2.99e-10 | 7.33e-07        |
| Humoral immune response                                               | 4.34e-10 | 7.99e-07        |
| Lymphocyte mediated immunity                                          | 1.35e-08 | 1.85e-05        |
| Phagocytosis recognition                                              | 1.51e-08 | 1.85e-05        |
| Phagocytosis                                                          | 9.74e-08 | 0.0001          |
| B-cell receptor signaling pathway                                     | 1.13e-07 | 0.0001          |
| Membrane invagination                                                 | 2.19e-07 | 0.0001          |
| Regulation of humoral immune response                                 | 2.57e-07 | 0.0001          |
| Positive regulation of B-cell activation                              | 4.37e-07 | 0.0002          |
| Adaptive immune response based on somatic recombination of            | 5.19e-07 | 0.0003          |
| immune receptors built from immunoglobulin superfamily domains       |          |                 |
| Defense response to bacterium                                         | 3.16e-06 | 0.0017          |
| FC receptor mediated stimulatory signaling pathway                    | 5.15e-06 | 0.0027          |
| Regulation of B-cell activation                                       | 6.38e-06 | 0.0031          |
| FC epsilon receptor signaling pathway                                 | 2.12e-05 | 0.0097          |
| Cell recognition                                                      | 2.37e-05 | 0.0102          |
| Positive regulation of cell activation                                | 3.69e-05 | 0.0151          |
| Activation of immune response                                         | 5.34e-05 | 0.0206          |
| Adaptive immune response                                              | 8.17e-05 | 0.0300          |
| Leukocyte migration                                                   | 0.0001   | 0.0482          |
Table 3. Biological pathways showing the most significant alterations in gene expression (Reactome)

| Sets                                                                 | P-value   | FDR corrected P |
|----------------------------------------------------------------------|-----------|-----------------|
| Scavenging of heme from plasma                                       | 5.06e-09  | 7.58e-06        |
| Creation of C2 and C4 activators                                     | 1.00e-07  | 7.49e-05        |
| Uptake/binding of ligands by scavenger receptors                     | 1.56e-07  | 7.84e-05        |
| Initial triggering of complement                                      | 2.55e-07  | 9.58e-05        |
| CD22 mediated BCR regulation                                          | 3.99e-07  | 0.0001          |
| FCGR activation                                                       | 1.05e-06  | 0.0002          |
| Role of LAT2 NTAL lab on calcium mobilization                        | 1.31e-06  | 0.0002          |
| Role of phospholipids in phagocytosis                                | 3.94e-06  | 0.0007          |
| Antigen activates B-cell receptor BCR leading to generation of second messengers | 5.65e-06  | 0.0007          |
| FCERI mediated CAPLUS2 mobilization                                 | 5.65e-06  | 0.0007          |
| Complement cascade                                                    | 5.73e-06  | 0.0007          |
| FCERI mediated MAPK activation                                       | 6.16e-06  | 0.0007          |
| Regulation of actin dynamics for phagocytic cup formation             | 5.73e-05  | 0.0066          |
| Cell surface interactions at the vascular wall                        | 7.21e-05  | 0.0077          |
| FCERI mediated NF KB activation                                      | 0.0001    | 0.0155          |
| FCGAMMA receptor FCGR dependent phagocytosis                         | 0.0002    | 0.0205          |