# Correlation Between Immunological Laboratory Status of Omega-3-Fatty-Acid-Supplemented Patients of a German Fertility Centre and Outcome of IVF-Treatment

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**Abstract:** A well-defined group of patients of a German Fertility Centre, all supplemented periconceptually with omega-3-fatty acids, was monitored for specific alterations in the immunological laboratory status in correlation with the outcome of in-vitro-fertilization-(IVF)-treatment. For 36 of 52 IVF-patients IVF-treatment was successful. Patients with IVF-success revealed statistically significant lower soluble interleukin-2-receptor concentrations than unsuccessfully treated patients. Decreased numbers of CD4-positive T-cells could be correlated with increased failure rates of IVF, increased numbers of regulatory T-cells on the other hand with significantly improved success rates. Six of the parameters analyzed (T-lymphocytes, T-helper cells, B-lymphocytes, CD4/CD8 ratio and both classes of regulatory T-cells) significantly more often were below the median of the respective distribution in patients without IVF-success than in successfully treated. These results offer the possibility of developing a simple prognostic measure for the outcome of an IVF-therapy by assessing these parameters in relation to the median of the respective distribution. With increasing numbers of parameters lying below the median, the probability of successful IVF-treatment is decreasing.

**Keywords:** Omega-3-Fatty Acid Supplementation, In Vitro Fertilization, Immunological Laboratory Status

## 1. Introduction

The shift of family planning into later stages of life is correlated with physiologically decreasing fertility. This is one of the main reasons for rising demand for reproductive medical treatments [1] with a mean age of 38.52 years for women and men [2]. The probability of successful IVF-treatment decreases with decreasing fertility [1]. Deeper understanding of this phenomenon on a mechanistic basis with the aim to develop optimization strategies for such elaborate treatments would enable to reduce physical, psychological and financial loads of affected couples.

So far, various research approaches have been investigating the influence of life-style modifications, especially dietary supplements, on fertility and miscarriage rates [3-4].

With respect to miscarriages chromosomal aberrations are described to cause up to 50% [5]. Reasons of anatomical, haematological and immunological nature have also been discussed as possible triggers for spontaneous miscarriages [6-7]. So, the meaning of red blood cells (RBC) for the onset of pregnancy is so far unclear. Maternal RBCs can be detected from day 10 after conception intravilliously within

the placenta, maternal blood flow is detected from pregnancy week 29 on [8]. Platelets seem to play an important role as early as from implantation on on [9]. A correlation of the innate and adaptive immune system with complications of pregnancy like spontaneous miscarriages, intrauterine growth retardation, pre-eclampsia and premature births has been shown [10]. During normal pregnancy women reveal a phenotypical suppressive immune profile, in premature births this can be different. Blidaru observed an elevation of B-lymphocytes, an increased CD4/CD8-ratio and diminished CD8-T-lymphocyte numbers in women with premature births [11]. Pre-eclampsia is associated with fetal growth retardation and increased maternal mortality [12]. Pre-eclampsia patients show reduced dioxygenase levels and an increased number of neutrophilic granulocytes [13-17]. Neutrophilic granulocytes and the synthesis of neutrophilic extracellular substances seem to have importance for as well the onset and the course of pregnancy [18]. The release of nitric oxide by neutrophilic granulocytes and their arginase-I activity are involved in the immune response regulation of T-cell activities [19]. Monocytes and macrophages also play important physiological roles in pregnancy. Upregulation of chemoattractant expression during periimplantantation and in the first phase of pregnancy reveal their significance [20]. Quantitative alterations of macrophages and monocytes during normal, uncomplicated pregnancy indicate their importance for fighting infections as well as for elimination of apoptotic placental cells [21]. Increased monocyte infiltration of uterine mucosa was found to be associated with pregnancy complications [22-23]. Natural killer cells were detected to be important for becoming pregnant and the onset of pregnancy [24-28]. Increased NK-cell numbers are considered as a trigger for spontaneous abortions [29]. T-lymphocytes also seem to be essentially involved in pregnancy and it´s onset. CD4+ und CD8+ T-lymphocytes influence implantation as well as embryonic development in a positive manner and provide a balance between trophoblasts and decidual tissue [30]. CD4+ und CD25+ positive T-cells are also involved in a normal [31-33]. Apparently different compounds of the innate as well as adaptive immune systems play important roles within the normal course of pregnancy as well as it´s complications [34-35].

Based on the data above we focussed on the availibility of a well-characterized group of omega-3-fatty acid supplemented patients in a German Fertility Centre in order to examine in a first preliminary approach the influence of immunological parameters on fertility, in particular on probabilities of becoming pregnant and the prevention of miscarriages. Hematological analysis focussed on quantitation of T-lymphocytes, T-helper and T-suppressor cells, CD4/CD8-ratio, NK-cells, B-lymphocytes, T-NK-cells and regulatory T-cells. Quantitative analysis of soluble interleukin-2 receptor (sIL-2R), interleukin-6, -8, -10, tumor necrosis factor-$\alpha$ (TNF-$\alpha$), immunoglobulins E, A, M, G and immunoglobulin G subclasses was performed. Based on these findings we evaluated if singular parameters or a combination of several of these parameters may be predictive for IVF-success under omega-3-fatty acid supplementation.

# 2. Material and Methods

## 2.1. Patients

Patients of a German Fertility Centre (Interdisziplinäres IVF-Zentrum Düsseldorf, Germany) with at least 2 miscarriages or non-successful IVF-therapies were included in the study. This was a patient group preselected with respect to negative preconditions for successful in vitro fertilization therapy. Patients with prednisolone-therapy and patients rejecting disclosure of their data were excluded.

A total of 52 patients with periconceptual omega-3-fatty acid supplementation were screened for a correlation between immunological status and IVF-success. 36 of 52 fertility patients became pregnant, one of these patients suffered from a miscarriage within the further course of pregnancy [3].

## 2.2. Blood Sampling

During omega-3-fatty acid supplemented IVF-treatment and the follow-up period blood sampled were drawn at in average 4 different endpoints. The mean interval between blood sampling was 2 weeks, the first sample was collected before supplementation started. EDTA-blood was used for flow cytometry analysis, lithium heparine plasma for the analysis of TNF-$\alpha$, serum for immunoglobulins, immunoglobulin subclasses, soluble interleukin-2 receptor and interleukins.

## 2.3. Flow Cytometry

Flow cytometry was performed by using a FACSCaliburTM (Becton Dickinson, Heidelberg, Germany, software BD Multiset) at laser wave lengths 635 and 488 nm for the analysis of T-lymphocytes (CD3+), T-helper cells (CD4+), T-suppressor cells (CD8+), CD4/CD8-ratio, NK-cells (CD16+, CD56+), T-NK-cells (CD56+, CD16+, CD3+), B-lymphocytes (CD19+), regulatory T-cells (CD4+, CD25+ / CD4+, CD25+, CD127-). 50 μl EDTA-blood was incubated for 15-30 minutes with 20 μl of the corresponding antibody mixture 1 (anti-CD3, anti-CD8, anti-CD45, anti-CD4) and antibody mixture 2 (anti-CD3, anti-CD16, anti-CD56, anti-CD45, anti-CD19) (BD Multiset IMK Kit). Immediately before analysis 450 μl of lysis solution (BD Multiset IMK Kit Lysis Solution) was added to each sample and incubated for 15-30 minutes.

For analysis of regulatory T-cells (CD4+, CD25+, CD127-) 50 μl EDTA-blood was incubated with 10 μl of the corresponding antibody in the dark, after 15-30 minutes 450 μl lysis solution was added for a further 15-30 minute incubation before analysis.

## 2.4. Enzyme Immunoassays

Quantitative analysis of immunoglobulins and cytokines

was performed according to the manufacturer's instructions by means of the following commercially available chemiluminescence-based enzyme immunoassays: immunoglobulins E, A, M, G and immunoglobulin G subclasses (Siemens Advia Centaur XP), soluble interleukin-2 receptor (Siemens Immulite), interleukin-6, -8, -10 (Siemens Immulite), TNF-α (Siemens Immulite).

### 2.5. Statistics

Statistical analysis was performed by means of IBM SPSS Statistics (version 22). Mean values of two groups were compared by means of the two-sample-t-test. The Mann-Whitney-U-test was used to proof if two samples belong to the same distribution.

By means of the Spearman rank correlation analysis the strength and direction of the correlation of two variables was analyzed. One-way ANOVA analysis was used to verify if the mean values different of lymphocyte populations differ between the various blood sampling terms. By discrimination analysis it was analyzed if prognosis of IVF-success or imminent complications like f.e. miscarriages is possible by quantitative analysis of different lymphocyte populations.

## 3. Results

### 3.1. Lymphocyte Subpopulations, IVF-Success and Miscarriage Rates

During the treatment and observation period lymphocyte subpopulations of successfully and not successfully treated IVF-patients were analyzed (Table 1). The patient, who suffered from a miscarriage, was integrated in the group of not-successfully treated patients. Patients without IVF-success (n=17) showed significantly lower numbers of T-helper cells (mean difference -34.0%, p=0.009), T-lymphocytes (mean difference -26.0%, p=0.015) and B-lymphocytes (mean difference -22.1%, p=0.063). Regulatory T-cells (CD4+, CD25+) were lower in patients without IVF-success as in successfully treated patients, but of marginal statistical significance.

*Table 1. Differences between lymphocyte subpopulations of omega-3-fatty acid supplemented patients with (n=35) and without IVF-success (n=17).*

| parameter | patients without IVF-success (n=17) | patients with IVF-success (n=35) | difference of the mean | p-value |
|---|---|---|---|---|
| B-lymphocytes (CD19+) | 236.5±165.7 | 303.3±194.5 | -66.8 | 0.063 |
| CD4/CD8-ratio | 84±145.2 | 107.2±138.7 | -23.2 | 0.378 |
| NK-cells (CD16+, CD56+) | 264.9±155.4 | 244.4±116.8 | 20.5 | 0.379 |
| regulatory T-cells (CD4+, CD25+) | 306.2±218.8 | 413.9±331.1 | -107.6 | 0.071 |
| regulatory T-cells (CD4+, CD25+) [%] | 16.5±9.3 | 17.3±6.8 | -0.8 | 0.553 |
| regulatory T-cells (CD4+, CD25+, CD127–) | 188.8±137.9 | 217.2±144.8 | -28.4 | 0.294 |
| regulatory T-cells (CD4+, CD25+, CD127–) [%] | 10.4±4.8 | 9.4±3.4 | 0.9 | 0.178 |
| T-helper cells (CD4+) | 714.8±379.9 | 1082.6±795.2 | -367.8 | 0.009 |
| T-lymphocytes (CD3+) | 1237.9±556.3 | 1673.5±1002.7 | -435.6 | 0.015 |
| T-NK-cells (CD56+, CD16+, CD3+) | 52.0±57.9 | 52.9±56.7 | -0.8 | 0.938 |
| T-suppressor cells (CD8+) | 485.6±216.1 | 548.9±282.6 | -63.3 | 0.218 |

Furthermore, lymphocyte populations of successfully treated omega-3-fatty acid supplemented IVF-patients (n=35) and the patient who suffered from a miscarriage after initial IVF-success (n=1) were compared (Table 2). The patient with the miscarriage showed significantly higher numbers of T-suppressor cells (mean difference +67,7%; p = 0,001), B-lymphocytes (mean difference +77,4%; p = 0,002), T-lymphocytes (mean difference +70,8%; p = 0,003), T-helper cells (mean difference +70,7%; p = 0,015), regulatory T-cells (CD4+, CD25+, CD127–) (mean difference +56,1%; p = 0,032) and regulatory T-cells (CD4+, CD25+) (mean difference +63,6%; p = 0,044). The CD4/CD8-ratio was also significantly elevated (mean difference +150%; p = 0,004). However, the miscarriage patient revealed significantly lowered numbers of NK-cells (mean difference -38.2%; p = 0,035). For T-NK-cells no differences were observed.

*Table 2. Differences between lymphocyte subpopulations of omega-3-fatty acid supplemented patients with IVF-success and the patient, who after initial IVF-success, suffered from a miscarriage.*

| parameter | patients without IVF-success (n=1) | patients with IVF-success (n=35) | difference of the mean | p-value |
|---|---|---|---|---|
| B-lymphocytes (CD19+) | 294.0±192.6 | 521.73±80.3 | -227.7 | 0.002 |
| CD4/CD8-ratio | 101.1±135.0 | 252.7±157.0 | -151.6 | 0.004 |
| NK-cells (CD16+, CD56+) | 248.2±117.4 | 153.4±43.4 | 94.8 | 0.035 |
| regulatory T-cells (CD4+, CD25+) | 403.5±332.6 | 660.1±165.3 | -256.6 | 0.044 |
| regulatory T-cells (CD4+, CD25+) [%] | 17.3±6.9 | 18.6±4.0 | -1.3 | 0.628 |
| regulatory T-cells (CD4+, CD25+, CD127–) | 212.4±144.7 | 331.6±93.9 | -119.2 | 0.032 |
| regulatory T-cells (CD4+, CD25+, CD127–) [%] | 9.4±3.5 | 9.3±2.2 | 0.1 | 0.914 |
| T-helper cells (CD4+) | 1052.5±797.7 | 1796.3±104.8 | -734.8 | 0.015 |
| T-lymphocytes (CD3+) | 1626.9±996.5 | 2778.4±172.8 | -1151.5 | 0.003 |
| T-NK-Zellen (CD56+, CD16+, CD3+) | 51.8±56.6 | 78.6±56.8 | -26.8 | 0.222 |
| T-suppressor cells (CD8+) | 534.3±278.3 | 896.0±110.4 | -361.7 | 0.001 |

### 3.2. Interleukins, TNF-α, Immunoglobulins and IVF-Success or Miscarriage Rates of Omega-3-Fatty Acid Supplemented Patients

Omega-3-fatty acid supplemented fertility patients without IVF-success revealed statistically significant higher soluble interleukin-2-receptor concentrations (536.1±271.7 IU/ml, p<0.01) than patients with IVF-success (416.8±169.4 IU/ml). The patient who suffered from a miscarriage during pregnancy revealed the highest sIL-2 receptor concentrations (816.9±84.8 IU/ml) (Figure 3). As a correlation of sIL-2R-levels with IVF success was significant in the group studied, a larger study is planned, based on the well-defined patient group treated in the German Fertility Centre.
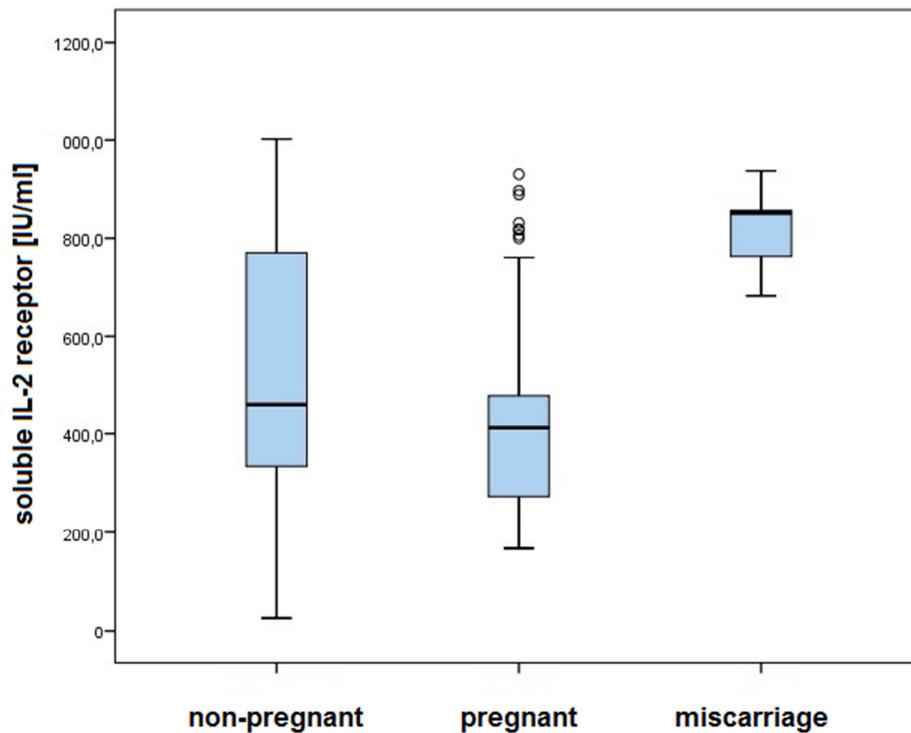


*Figure 1. Correlation of the amount of soluble IL-2 receptor and the outcome of IVF-treatment of omega-3-fatty acid supplemented patients.*

No significant differences were found for IL-6-, IL-8-, IL-10- and TNF-α-concentrations during the observation period between both patients groups (Table 3). Successfully treated IVF-patients showed IL-6 concentrations lowered by more than factor 10. Due to the significant spread of value distribution in non-pregnant patients this finding was statistically not significant. IL-8 concentrations in not-successfully treated patients were nearly twice as high compared to those not-successfully treated. In both groups the spread of value distribution was rather high, so the differences were not significant.

*Table 3. Comparison of IL-6, IL-8, IL-10 und TNF-α concentrations in omega-3-fatty acid supplemented fertility patients with (n=35) and without (n=17) IVF-success.*

| parameter | patients without IVF-success (n=1) | patients with IVF-success (n=35) | difference of the mean | p-value |
|---|---|---|---|---|
| IL-6 [pg/ml] | 33.8±89.8 | 2.1±0.4 | 31.7 | 0.107 |
| IL-8 [pg/ml] | 14.8±22.9 | 27.6±59.4 | -12.8 | 0.539 |
| IL-10 [pg/ml] | 5.0±0.1 | 6.7±7.4 | -1.6 | 0.518 |
| TNF-α [pg/ml] | 5.6±0.9 | 6.7±1.7 | -1.2 | 0.137 |

Immunoglobulin A, E, M, G and Ig G subclasses were analyzed for 30 randomly selected fertility patients. 20 of these patients (66.7%) became pregnant, no patient suffered from a miscarriage. Fertility patients without IVF-success revealed higher immunoglobulin E levels, but this finding was statistically not significant. IgA, M, G and IgG-subclass concentrations revealed no differences between both groups (Table 4).

*Table 4. Comparison of immunoglobulin concentrations in omega-3-fatty acid supplemented fertility patients with (n=35) and without (n=17) IVF-success.*

| parameter | patients without IVF-success (n=1) | patients with IVF-success (n=35) | difference of the mean | p-value |
|---|---|---|---|---|
| IgA [mg/ml] | 1.7±0.9 | 2.1±0.7 | -0.38 | 0.277 |
| IgE [ng/ml] | 68.1±105.4 | 55.7±59.9 | 12.5 | 0.672 |
| IgG [mg/ml] | 9.3±3.6 | 11.5±2.2 | -1.6 | 0.115 |
| IgG1 [mg/ml] | 5.2±2.1 | 6.4±1.4 | -1.1 | 0.123 |
| IgG2 [mg/ml] | 3.6±1.6 | 4.7±1.4 | -1.2 | 0.107 |

| parameter | patients without IVF-success (n=1) | patients with IVF-success (n=35) | difference of the mean | p-value |
|---|---|---|---|---|
| IgG3 [mg/ml] | 0.4±0.2 | 0.6±0.3 | -0.2 | 0.109 |
| IgG4 [mg/ml] | 0.3±0.5 | 0.7±0.9 | -0.3 | 0.369 |
| IgM [mg/ml] | 1.2±0.8 | 1.2±0.5 | 0.1 | 0.809 |

### 3.3. Discrimination Analysis

In order to examine if the parameters analyzed are suitable to predict IVF-success of omega-3-fatty acid supplemented fertility patients, discrimination analysis was performed. In the first step analysis was performed with the variable "pregnant" and "not-pregnant" after IVF-treatment. Explanatory variables were absolute numbers of T- and B-lymphocytes, NK-cells, T-helper cells, T-suppressor cells, T-NK-suppressor cells, T-NK-cells, regulatory T-cells, CD4/CD8-ratios, soluble interleukin-2-receptor and the age of the patient. The following discriminant equation (Figure 4) could be derived:

$$
\begin{aligned}
D = &-8{,}332 - 0{,}001 \cdot (T - lymphocytes_{CD3+}) - 0{,}001 \cdot (B - lymphocytes_{CD19+}) + 0{,}002 \cdot \left(NK - cells_{CD16+,CD56+}\right) \\
&+0{,}002 \cdot (T - helper\ cells_{CD4+}) + 0{,}001 \cdot (T - suppressor\ cells_{CD8+}) - 0{,}002 \cdot \left(\frac{CD4}{CD8} - ratio\right) \\
&-0{,}001 \cdot \left(T - NK - cells_{CD56+,CD16+,CD3+}\right) + 0{,}000 \cdot \left(regulatory\ T - cells_{CD4+,CD25+,CD127-}\right) \\
&+0{,}000 \cdot \left(regulatory\ T - cells_{CD4+,CD25+}\right) + 0{,}192 \cdot age + 0{,}003 \cdot (soluble\ interleukin - 2 - receptor)
\end{aligned}
\tag{1}
$$

By this grouping of 89% could be performed correctly (Table 5). A non-correct assignment occurred for blood profiles of 15 non-pregnant patients who were erroneously classified as pregnant, whereas blood profiles of 3 pregnant patients were erroneously assigned non-pregnant.

**Table 5.** *Results of discrimination analysis using numbers of T- and B-lymphocytes, NK-cells, T-helper, T-suppressor-, T-NK-cells, regulatory T-cells, CD4/CD8-ratios, soluble IL-2 receptor and age of patients for the variables "pregnant" and "non-pregnant".*

| | non-pregnant (predicted) | pregnant (predicted) |
|---|---|---|
| non-pregnant (true) | 9 | 15 |
| pregnant (true) | 3 | 137 |

This was followed by discrimination analysis with the variables "pregnant" and "miscarriage" after IVF-treatment by using the absolute numbers of T- and B-lymphocytes, NK-cells, T-helper and T-suppressor cells, T-NK-cells, regulatory T-cells, CD4/CD8-ratio, soluble interleukin-2 receptor and the age of the patient. The following discriminant equation (Figure 5) could be derived:

$$
\begin{aligned}
D = &1{,}680 + 0{,}002 \cdot (T - lymphocytes_{CD3+}) + 0{,}003 \cdot (B - lymphocytes_{CD19+}) - 0{,}002 \cdot \left(NK - cells_{CD16+,CD56+}\right) \\
&-0{,}003 \cdot (T - helper\ cells_{CD4+}) - 0{,}002 \cdot (T - suppressor\ cells_{CD8+}) + 0{,}001 \cdot \left(\frac{CD4}{CD8} - ratio\right) + \\
&0{,}004 \cdot \left(T - NK\ cells_{CD56+,CD16+,CD3+}\right) - 0{,}001 \cdot \left(regulatory\ T - cells_{CD4+,CD25+,CD127-}\right) \\
&+0{,}000 \cdot \left(regulatory\ T - cells_{CD4+,CD25+}\right) - 0{,}109 \cdot age + 0{,}004 \cdot (soluble\ interleukin - 2 - receptor)
\end{aligned}
\tag{2}
$$

By this grouping of 99.3% could be performed correctly (Table 6). The blood-profile of one patient was erroneously classified as "miscarriage-profile", whereas the actual patient suffering from a miscarriage was classified correctly. 7 cases could not be classified.

So, a specific constellation of the different lymphocyte-parameters seems to emerge for miscarriage.

**Table 6.** *Results of discrimination analysis using numbers of T- and B-lymphocytes, NK-cells, T-helper, T-suppressor-, T-NK-cells, regulatory T-cells, CD4/CD8-ratios, soluble IL-2 receptor and age of patients for the variables "pregnant" and "miscarriage".*

| | pregnant (predicted) | Miscarriage (predicted) |
|---|---|---|
| pregnant (true) | 139 | 1 |
| miscarriage (true) | 0 | 7 |

Based on these findings of discrimination analysis it may be postulated that there exists a specific immunological blood profile for each patient that should potentially allow to predict success of IVF-treatment.

As the application of discriminant analysis is rather complex and therefore not suitable for every day practice, our goal was to develop a simple algorithm based on the finding of blood parameters analyzed so far, that enables a prognosis for the further course of pregnancy in fertility patients undergoing IVF-treatment.

For this purpose medians of the different blood parameters were calculated and the data grouped in relation to the

corresponding median (Table 7). Values obtained in such a way were put into relation to the outcome of IVF-fertility treatment (pregnant, non-pregnant, miscarriage).

Independency of the position of blood parameters from IVF-treatment success was examined by the Chi-square test of independence.

***Table 7.*** *Comparison of position of the parameters analyzed in relation to the median of the sample in dependence of gravidity.*

| parameter | gravidity | below median | median | above median | p-value |
|---|---|---|---|---|---|
| T-lymphocytes (CD3+) | non-pregnant | 21 | 1 | 12 | 0.024 |
| | pregnant | 81 | 1 | 84 | |
| | miscarriage | 0 | 0 | 7 | |
| NK-cells (CD16+, CD56+) | non-pregnant | 14 | 0 | 20 | 0.224 |
| | pregnant | 82 | 3 | 81 | |
| | miscarriage | 6 | 0 | 1 | |
| T-helper cells (CD4+) | non-pregnant | 23 | 0 | 11 | 0.017 |
| | pregnant | 79 | 2 | 85 | |
| | miscarriage | 0 | 0 | 7 | |
| B-lymphocytes (CD19+) | non-pregnant | 23 | 0 | 11 | 0.003 |
| | pregnant | 80 | 0 | 85 | |
| | miscarriage | 0 | 0 | 7 | |
| T-NK-cells (CD56+, CD16+, CD3+) | non-pregnant | 18 | 0 | 16 | 0.001 |
| | pregnant | 85 | 1 | 80 | |
| | miscarriage | 0 | 1 | 6 | |
| T-suppressor-cells (CD8+) | non-pregnant | 17 | 1 | 16 | 0.015 |
| | pregnant | 86 | 0 | 80 | |
| | miscarriage | 0 | 0 | 7 | |
| CD4/CD8-ratio | non-pregnant | 25 | 0 | 9 | 0.004 |
| | pregnant | 78 | 1 | 87 | |
| | miscarriage | 0 | 0 | 7 | |
| regulatory T-cells (CD4+, CD25+) | non-pregnant | 22 | 0 | 12 | 0.032 |
| | pregnant | 81 | 2 | 83 | |
| | miscarriage | 0 | 0 | 7 | |
| regulatory T-cells (CD4+, CD25+, CD127–) | non-pregnant | 23 | 0 | 11 | 0.014 |
| | pregnant | 78 | 3 | 85 | |
| | miscarriage | 0 | 0 | 7 | |
| soluble IL-2 receptor | non-pregnant | 11 | 0 | 13 | 0.082 |
| | pregnant | 74 | 2 | 65 | |
| | miscarriage | 0 | 0 | 7 | |

For T-lymphocytes (CD3+), T-helper-cells (CD4+), B-lymphocytes (CD19+), CD4/CD8-ratios, regulatory T- (CD4+, CD25+) and regulatory T-cells (CD4+, CD25+, CD127–) numbers of non-pregnant patients were twice as often below the median of the sample than above. For pregnant patients the ratio was balanced. For the patient with the miscarriage during a later course of pregnancy all numbers were above the median of the sample. For NK-cells (CD16+, CD56+) and soluble interleukin-2-receptor no differences were found in both patient groups. As for T-lymphocytes, T-helper cells, B-lymphocytes, CD4/CD8-ratio and regulatory T-cells a value below the corresponding median was associated with a negative outcome of IVF-success, in the next step a variable was developed in order to indicate how many of one patients´ parameters were below the median of the sampling distribution (Table 8).

***Table 8.*** *Dependence of IVF-success on the numbers of lymphocytes below the median of the respective sampling distribution.*

| number of values below median | patients without IVF-success (n=17) | patients with IVF-success (n=35) | number of pregnant patients | cumulative proportion of pregnant patients |
|---|---|---|---|---|
| 0 | 1 | 43 | 97.7 | 97.7 |
| 1 | 3 | 26 | 89.7 | 94.5 |
| 2 | 5 | 16 | 76.2 | 90.4 |
| 3 | 5 | 16 | 76.2 | 87.8 |
| 4 | 1 | 19 | 95.0 | 88.9 |
| 5 | 9 | 19 | 67.9 | 85.3 |
| 6 | 10 | 34 | 77.3 | 83.6 |

Patients without IVF-success presented significantly more values below the median of the sampling distribution than successfully treated patients (p = 0,007; Figure 4). With increasing numbers of values below the median the cumulative share of pregnant patients decreased highly significant (r=-0,966; p < 0,001).

This is a simple measure which can be easily determined for each fertility patient. For this purpose measured numbers for six parameters T-lymphocytes (CD3+), T-helper cells (CD4+), B-lymphocytes (CD19+), CD4/CD8-ratios, regulatory T- (CD4+, CD25+) and regulatory T-cells (CD4+, CD25+, CD127–) are compared with the respective median of the distribution. With increasing numbers of values below the median the probability of becoming pregnant after IVF-

treatment decreases.

The stronger the correlation between the sample of IVF-patients and the outcome of IVF-treatment, the more precise the actual median can be determined and the likelihood of IVF-treatment success predicted.

# 4. Discussion

Influence of lymphocytes on IVF-success

In our study omega-3-fatty acid supplemented patients without IVF-success revealed decreased numbers of T-helper cells, T- and B-lymphocytes, CD4/CD8-ratio and regulatory T-cells. So far it has been described that a change in the relationship of TH1- (cell-mediated) and TH2-helper cells (humoral immune response) is correlated with an implantation failure of in-vitro-fertilized embryos [36]. Altered TH1/TH2-ratios led to repetitive failures of IVF-attempts. Hormone application during fertility treatment even increases the negative impact of high TH1/TH2-ratios [37-39]. Patients with recurrent spontaneous abortions revealed significantly increased TH1/TH2-ratios [38]. Additionally decreased numbers of CD4-positive T-cells were associated with unsuccessful IVF-treatments [40] as well as increased numbers of NK-cells [6, 41-42]. For the latter a tendency could be shown in our study: patients without IVF-success revealed increased NK-cell numbers, but these were statistically not significant.

Recently published data indicate a correlation of increased numbers of NK- and T-NK-cells and a significant improvement of IVF-success rates [43-44]. Therefore data on the impact of NK-cells are contradictory and not to be clarified further by our study. On the other hand we found increased numbers of regulatory T-cells in successfully IVF-treated patients, concordant with literature: increased concentrations of hemeoxygenase-1 lead to an increase in regulatory T-cells and therefore to a promotion of fertilization, implantation and fetal growth [45]. Stimulation by human chorionic gonadotropin increases numbers of regulatory T-cells and leads to increased IVF-success-rates [46]. Increased numbers of regulatory T-cells are unequivocally related with increased IVF-success [47]. In our omega-3-fatty acid supplemented patient cohort patients without IVF-success showed slightly, but statistically not significant different B-lymphocyte numbers compared with IVF-success patients. Data published so far on B-lymphocytes are contradictory [48].

Influence of lymphocytes on miscarriage rates

In our study one patient suffered from a miscarriage during the course of pregnancy. Compared to pregnant fertility patients this patient showed significantly increased numbers of T-suppressor cells, B-lymphocytes, T-lymphocytes, T-helper and regulatory T-cells, additionally a significant increase of CD4/CD8-ratio and a significant decrease of NK-cells. This is not concordant with data published so far [38, 49-51]. As the miscarriage rate in this study was low, not further interpretation is possible. On the other hand a favourable effect of omega-3-fatty acid supplementation on

prevention of miscarriages in IVF-patients can most likely to be extrapolated [3].

Prognostic value of the size of different lymphocyte populations

In this study T- and B-lymphocytes, T-helper cells, CD4/CD8-ratios and regulatory T-cells of patients without IVF-success were significantly below the median of the corresponding distribution compared to successfully treated fertility patients. Increasing numbers of values below the median were correlated with a highly significant decrease of the cumulative ratio of pregnant patients.

An approach using blood profiles of fertility patients in order to give a prognosis for the further course of pregnancy was published recently [40]. Increased expression of CD56 and CD158a in T-lymphocytes, decreased numbers of CD4-positive T-cells, increased expression of HLA-DR in T-suppressor and NK-cells, increased numbers of NK-cells, increased expression of CD158a and decreased expression of CD8 in NK-cells were described as negative prognostic markers for the further course of fertility treatment. In this study, similar to our data, the number of parameters in a critical range were determined for each patient. With increasing numbers of parameters the probability of IVF-success decreased [40]. Additionally it was shown that the combination of critical blood parameters was of better prognostic value than the use of single parameters. Summarizing, the definition of thresholds, below which a negative impact on pregnancy is evident, seems to be reasonable when defining risk profiles for each patient. In the study presented here the median of different immunological blood parameters of the respective distribution was defined as this threshold. This first approach could be refined in further studies with increasing size and therefore improve prognosis for the outcome of elaborate and expensive IVF-treatments.

Influence of immunoglobulins and interleukins on IVF-success

Omega-3-fatty acid supplemented, successfully IVF-treated fertility patients revealed significantly decreased concentrations of soluble interleukin-2-receptor than those patients without IVF-success. Data published so far showed no unequivocal impact of omega-3-fatty acid supplementation on sIL-2R serum levels, some studies describe no influence [52-53], others a decrease of serum levels [54-55]. The level of sIL-2R could not be correlated with the risk of ectopic pregnancies [56-57]. But increased levels were recognized as negative prognostic criteria for the success of IVF-treatments [58]. This finding could be confirmed by our study as not only high levels were correlated without IVF-success but also with the only miscarriage observed in the study group. This encourages us to use sIL-2R as a regular screening marker within the treatment period of patients in the Fertility Centre. Increased levels in successfully treated IVF-patients imply intensified monitoring in order to prevent an impending miscarriage.

For immunoglobulins A, E, G and M as well as immunoglobulin G subclasses, interleukins-6, -8, -10 and

tumor necrosis factor-α no significant differences were found between both patient groups of our study. Data published so far on immunoglobulin levels were mostly based on small patient numbers. Fertility patients with antiphospholipid syndrome showed higher concentrations of immunoglobulin G and M [59] as well as immunoglobulin A [60] than patients with normal fertility.

Data on interleukins are inconsistent. Serum levels of interleukin 6, 8 and tumor necrosis factor-α were described to be increased in patients with ectopic pregnancy [56], but some studies also showed decreased interleukin-8 levels [57]. Contradictory to our data is the finding of an increased ratio of TNF-α and IL-10 on infertile compared to fertile patients [61].

## 5. Conclusion

A correlation of decreased numbers of CD4-positive T-cells with increased failure rates of IVF-treatments was shown by us in accordance to data published so far, this also applies to increased numbers of regulatory T-cells and significantly improved success rates.

For six of the parameters analyzed (T- and B-lymphocytes, T-helper cells, CD4/CD8-ratios and both classes of regulatory T-cells) numbers were significantly more frequently below the median of the corresponding distribution for patients without IVF-success than for successfully treated patients. Depression of CD4-positive T-cells was associated with increased failure rates of IVF-treatments. Increased numbers of regulatory T-cells led to significantly improved perspectives of IVF-treatments. For T- and B-lymphocytes, T-helper cells, CD4/CD8-ratio and regulatory T-cells the numbers of patients without IVF-success were significantly more often below the median of the corresponding distribution than in successfully treated patients. These findings enable to determine a simple prognostic measure for the further course of pregnancy by comparing the numbers of these six parameters with the corresponding median. Increasing numbers below the median can be correlated with decreasing IVF-success. Additionally, patients with IVF-success revealed statistically significant lower soluble interleukin-2-receptor concentrations than unsuccessfully treated patients. In our cohort of patients, the soluble interleukin-2 receptor turned out as a possible regular screening marker within the treatment period of patients in order to predict impending miscarriages.

## 6. Recommendations

Of practical relevance is a simple prognostic measure for the further course of a fertility patient´s pregnancy which can be established by comparing the T- and B-lymphocytes, T-helper cells, CD4/CD8-ratios and both classes of regulatory T-cells with the corresponding median of successfully treated patients. Additionally, soluble intereukin-2-receptor concentrations, which are easily to measure in clinical as well as in outpatient settings, may give additional information with respect to pregnancy outcome.

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