Human synovia contains trefoil factor family (TFF) peptides 1-3 although synovial membrane only produces TFF3: implications in osteoarthritis and rheumatoid arthritis

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Abstract: Objective: In the past we demonstrated that trefoil factor family peptide 3 (TFF3) supports catabolic functions in diseased articular cartilage widening the knowledge of the functional spectrum of TFF peptides. The findings revealed that TFF3 is a multifunctional peptide with the ability to link inflammation with tissue remodeling processes in articular cartilage and suggest that TFF3 is a factor in the pathogenesis of osteoarthritis (OA) and septic arthritis. As in joint physiology and diseases such as OA also the synovial membrane (SM) of the joint capsule plays a central role we here analyzed the ability of SM to produce and secrete TFF peptides and compared healthy SM as well as its secretion product synovial fluid (SF) with SM and SF from patients suffering from OA or rheumatoid arthritis (RA). Methods: RT-PCR, quantitative RT-PCR, Western blot and ELISA were used to measure the expression of TFF1, -2 and -3 in healthy SM and in SM from patients suffering from OA or RA. For tissue localization we investigated all three TFF peptides (TFF1-3) in differently aged human SM of healthy donors by means of immunohistochemistry. Results: Only TFF3 but not TFF1 and -2 was expressed in SM from healthy donors as well as tissue samples from patients with OA or RA on protein and mRNA level. In contrast, all three TFFs were detected in all samples of SF on protein level. No significant changes were observed for TFF1 at all. TFF2 was significantly upregulated in RA samples in comparison to OA samples. TFF3 protein was significantly downregulated in OA samples in comparison to healthy samples and in cases of RA significantly upregulated compared to OA. In contrast, in SM TFF3 protein was not significantly regulated. Conclusion: The data demonstrate the production of TFF3 in SM. Unexpectedly SF contains all three known TFF peptides. As neither articular cartilage nor SM produce TFF1 and TFF2 we speculate that these originate with high probability from blood serum. As TFF3 was not significantly regulated in SM samples under diseased conditions and the relative protein concentration of TFF3 was in total about 14-fold (healthy: 26-fold, OA: 6-fold, RA: 14-fold) lower in SM compared to SF we hypothesize that other joint structures like articular cartilage and diffusion from blood serum play a more important role in the regulation of TFF3 in healthy, OA and RA. Downregulation of TFF3 in SF but upregulation of TFF3 in SM (not statistically significant) in cases of OA suggests hypotheically the attempt of SM to compensate for a disturbed or changed TFF transport from the blood which is seemingly increased for TFF2 in case of RA.
**Keywords:** trefoil factor family peptides (TFF), synovial membrane (SM), synovial fluid (SF), osteoarthritis (OA), rheumatoid arthritis (RA)

1. Introduction

In vertebrates, joints have evolved into highly conserved organs that are essential for locomotion. Their joint capsules are lined with a synovial membrane (SM), which secretes synovial fluid (SF) and is therefore essential for maintaining the functionality of the joint connections. SM is the innermost membrane of the joint capsule in diarthroses consisting of an intimal 1-2 cells comprising layer and a subintimal layer containing particularly blood vessels, adipocytes, fibroblasts and macrophages [1]. The intimal layer comprises two different cell types: type A-cells (type-A-synoviocytes) that belong to macrophage lineage, repopulate from local precursors [2] and which main function is phagocytosis of debris from the joint cavity [3] and type B-cells (type-B-synoviocytes) which are fibroblast-like cells that produce and secrete hyaluronan [4], lubricin (PRG4) [5] and surface-active phospholipids [6] among others as essential components of SF. The SF primary functions as a biological lubricant, provides nutrients as well as regulatory cytokines and reduces friction and wear of the participating joint structures during movement [7].

The mammalian trefoil factor (TFF) family comprises three protease-resistant peptides of 7-12 kDa (TFF1), TFF2, and TFF3 [also ITF = intestinal trefoil factor] that have in common a distinct highly conserved motif of 6 cysteine residues, the so-called trefoil domain [8]. TFF peptides are mainly expressed in mucous epithelia (mucosa) performing cell type-specific interactions with mucins [9]. Primarily TFFs were described as secretory peptides of the gastrointestinal tract by several studies [10] but later were also found in various other tissues all over the body [11].

The functions of the three known TFF peptides are as diverse as the tissues producing them [12]: TFF peptides are important for maintenance and repair of intestinal mucosa. They influence the mucous viscosity [13] as well as the surface mucosal defense and protect the mucous barrier [14]. They induce cell migration and are therefore motogens in restitution, an important mechanism of epithelial repair, also in bone repair [15]. Furthermore, TFF peptides act anti-apoptotic, are involved in immune response [16], promote angiogenesis and have healing effects [for review see 17, 18].

In a previous study we found that TFF peptides are absent in healthy articular cartilage, but, in certain disease states such as osteoarthritis (OA) or septic arthritis, the TFF family member TFF3 supports catabolic functions and links inflammation with tissue remodeling processes, which distinguished TFF3 to be a potential factor in the pathogenesis of OA [19].

OA is a chronic degenerative joint disease characterized by cartilage degradation and the most common joint disease all over the world causing an increasing burden on the health system [20]. Inflammatory symptoms, especially synovitis, usually occur secondary during the progression of the disease. Rheumatoid arthritis (RA) is, in contrast to OA, a primary inflammatory joint disease characterized by chronic inflammation, destruction of articular cartilage and subarticular bone accompanied by the loss of joint function. About 0.5-1 % of the global population are affected and it is a major cause of disability [21]. SM and SF are involved in both, OA and RA. An overview on the role of synovitis in OA, for example production of pro-inflammatory cytokines [22] or synovial hypertrophy and hyperplasia [23], has been summarized by Sellam and Berenbaum [24]. Concerning RA, the SM is the main tissue to be affected by the disease [25] and synovial fibroblasts play a pivotal role in the pathogenesis [26].

With regard to the above mentioned findings we questioned in our present study whether TFF peptides are also expressed in SM and are secreted into SF and with that could play a role in the pathogenesis of OA and RA by being up- or downregulated under disease conditions in comparison to healthy tissues and SF.

2. Results
2.1. TFF3 but not TFF1 and TFF2 is expressed in human SM of healthy donors and in SM of patients suffering from OA or RA on the protein and RNA level

2.1.1. RT-PCR

Gene expression of TFF1, -2 and -3 of healthy, osteoarthritis (OA) and rheumatoid arthritis (RA) affected SM was analyzed by RT-PCR. Specific amplification products were only detected for TFF3 (≈ 302 bp) in all sample groups (healthy, OA and RA) whereas TFF1 and -2 were absent in either healthy nor OA or RA SM. mRNA of human stomach served as positive control for all three TFFs and β-actin as loading control.

Figure 1. Distribution of TFF peptides in synovial membrane (SM). A) RT-PCR analysis of TFF1, -2 and -3 mRNA in human SM of healthy (28, 48, 54, 78 and 92 years (1/2/3/4/5)), rheumatoid arthritis (RA) (6/7) and osteoarthritis (OA) (8/9) samples. Line 10 represents the negative control (without cDNA template) and human stomach severed as positive control (11). Beta-actin (β-actin) was used as loading control. B) Immunohistochemical analysis of TFF1, -2 and -3 in human SM of 22 (a, b, c), 48 (d, e, f) and 83 (g, h, i) year old healthy donors. TFF3 is present in each examined sample (c, f, i). TFF1 (a, d, g) and TFF2 (b, e, h) reveal negative results irrespectively of the age of the donor. Insets show magnifications. Scale bars [3]: 100 μm. Red staining indicates positive antibody reaction.

2.1.2. Immunohistochemistry

Immunohistochemistry was performed on 5μm sections of healthy human SM from three donors aged 22, 48 and 83 years. Using specific antibodies to TFF1, -2 and -3, reactivity in SM (indicated by a red reaction product) could be visualized only for TFF3 and was especially strong in Type B synoviocytes of SM. In addition, intense TFF3 immunoreactivity was also present in the walls of blood vessels within the subsynovial layer. Our
results did not show an obvious difference when comparing the slides of the three differently aged donors. No antibody reactivity was observed for TFF1 or TFF2 in any of the samples.

2.1.3. Real-time RT-PCR

TFF3 gene expression was analyzed in human SM from healthy donors and in samples of patients suffering from OA and RA by means of real-time RT-PCR. Our data revealed inter-individual differences within each sample group. Although the results showed a slight upregulation in the diseased samples comparing OA (Error! Reference source not found.2A) or RA (Error! Reference source not found.2B) with SM from healthy donors, this difference was not statistically significant.

Figure 2. Relative gene expression and relative protein concentration of TFF3 in human synovial membrane (SM). A) SM samples of healthy (n = 3) and osteoarthritis (n = 5) affected knee joints. No significant difference is detected between healthy and OA samples performing the Mann-Whitney U-test (significance level p ≤ 0.05). B) SM samples of healthy (n = 3) and rheumatoid arthritis (n = 5) affected knee joints. No significant difference is detected between healthy and RA samples performing the Mann-Whitney U-test (significance level p ≤ 0.05). C) ELISA of TFF3 in human SM of healthy (n = 10), OA (n = 10) and RA (n = 10) samples. Mean values are: 298.4 pg/mg (healthy), 473.7 pg/mg (OA), 299.3 pg/mg (RA). The protein concentration is expressed in pg/mg and visualized as mean value and standard error of the mean (SEM). The protein amount of TFF3 in the three groups showed no statistically significant difference performing the ANOVA with Bonferroni correction (significance level p ≤ 0.05).

2.1.4. ELISA

ELISA was only performed for TFF3 in SM as this was the only TFF peptide detectable in healthy or diseased SM. For this the protein amount of TFF3 in healthy human SM was measured and compared to SM of patients suffering from OA or RA revealing a protein amount of 298.4 pg/mg in the healthy group (ranging from 46.5 pg/mg to 663.0 pg/mg), 473.7 pg/mg (ranging from 141.1 pg/mg to 1125.3 pg/mg) in case of OA, and 299.3 pg/mg (ranging from 155.9 pg/mg to 701.8 pg/mg) in case of RA. Compared to the healthy samples the protein amount of TFF3 in RA was nearly the same whereas in OA it was 1.6-fold higher (not statistically significant) (Error! Reference source not found.2C).

2.2. All three TFF peptides (TFF1, -2 and -3) are detectable in healthy synovial fluid as well as in SF of patients suffering from OA or RA.
2.2.1. Western blot

Presence of TFF1, -2 and -3 was analyzed by Western blot in SF from healthy donors as well as in SF of patients suffering from OA and RA. Results revealed antibody reactivity in all samples including human stomach, which served as a positive control, for all three TFFs. Distinct protein bands were detected for TFF1 at around 55 kDa, for TFF2 and -3 at around 55 kDa, 37 kDa, 30 kDa, and 23 kDa (Error! Reference source not found.3A). Furthermore, TFF3 was detectable at about 14 kDa in a healthy as well as a RA sample matching the dimeric form of TFF3. Although we performed the Western blot under reducing conditions with DTT and β-ME as reducing agents we did not detect the monomeric forms of any of the three TFFs in SF samples, which would have resulted in protein bands at about 7 kDa (TFF1 and -3) or 12 kDa (TFF2).

2.3. TFF2 protein concentration is significantly increased in synovial fluid of patients suffering from RA, TFF3 protein concentration is reduced in cases of OA and RA, and TFF1 shows no change.

2.3.1. ELISA

ELISA was performed in order to measure the protein concentration of TFF1, -2 and -3 in healthy human SF and SF of patients suffering from OA or RA (Error! Reference source not found.3B-D). TFF1 was detected in all samples except for one case of healthy SF and was not significantly changed between healthy (50.9 pg/mg, ranging from 0.8 pg/mg to 258.1 pg/mg) and diseased conditions (OA: 31.2 pg/mg, ranging from 9.5 pg/mg to 117.3 pg/mg and RA: 33.0 pg/mg, ranging from 18.8 pg/mg to 67.7 pg/mg) (Error! Reference source not found.3B). TFF2, which was also present in all samples, revealed a low protein concentration in healthy samples (13.7 pg/mg, ranging from 2.4 pg/mg to 74.3 pg/mg) that was nearly not changed in cases of OA (9.0 pg/mg, ranging from 0.4 pg/mg to 45.9 pg/mg) but was strongly increased in cases of RA (203.5 pg/mg, ranging from 0.3 pg/mg to 835.4 pg/mg) (Error! Reference source not found.3C). The results show a 22.6-fold higher TFF2 protein concentration in RA in comparison to OA (statistically significant) and a 14.9-fold higher concentration in RA compared to healthy samples (not statistically significant). TFF3 was significantly downregulated (2.6-fold) in OA samples of SF (2833.9 pg/mg, ranging from 1730.8 pg/mg to 4828.8 pg/mg) in comparison to healthy ones (7807.0 pg/mg, ranging from 2214.6 to 29279.2 pg/mg). With regard to the relation of OA and RA, the relative protein concentration of TFF3 was significantly not as much (1.4-fold) upregulated in RA samples (4083.9 pg/mg, ranging from 2051.8 pg/mg to 8413.0 pg/mg) than in OA samples (2833.9 pg/mg, ranging from 1730.8 pg/mg to 4828.8 pg/mg) (Error! Reference source not found.3D). Comparison between all three TFF peptides revealed that the protein concentration of TFF3 in total was manifold higher than the concentration of TFF1 (127.8-fold) or TFF2 (65.1-fold).
Figure 3. Detection and quantification of TFF peptides in synovial fluid (SF). A) Western blot analysis of TFF1, -2 and -3 in human SF of healthy (1/2), osteoarthritis (OA) (3/4) and rheumatoid arthritis (RA) (5/6) samples. Proteins of human stomach serving as positive control (7) were included in the test. Actin (= 43 kDa) and alpha-1-antitrypsin (= 51 kDa) were used as loading control. Molecular weight marker is shown on the right. ELISAs of TFF1 (B), -2 (C) and -3 (D) in human SF of healthy (n = 13), OA (n = 20) and RA (n = 20) samples. Mean values are: TFF1: 50.87 pg/mg (healthy), 31.16 pg/mg (OA) and 33.04 pg/mg (RA); TFF2: 13.69 pg/mg (healthy), 8.97 pg/mg (OA), 203.50 pg/mg (RA); TFF3: 7807.29 pg/mg (healthy), 2833.90 pg/mg (OA), 4083.92 pg/mg (RA). The protein concentration is expressed in pg/mg and visualized as mean value and standard error of the mean (SEM). (*) indicates significant differences performing the Mann-Whitney U-Test (significance level p ≤ 0.05).

3. Discussion

In the past we described TFF3 production by human articular chondrocytes if the cartilage was affected by osteoarthritis (OA) whereas chondrocytes of healthy articular cartilage were TFF3 negative [19]. Furthermore, our findings showed that pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) induced TFF3 gene expression in cultured primary articular chondrocytes. In addition there was upregulation of distinct matrix metalloproteinases (MMP), which are well known as cartilage degrading enzymes in primary cultured chondrocytes after stimulation with recombinant human (rh) TFF3 [19]. Stimulation with rhTFF3 revealed a proapoptotic effect by increasing caspase 3/7 activity. These observations led to the conclusion that TFF3 supports catabolic functions in human articular cartilage and is a possible factor in the pathogenesis of OA. Bijelic et al. investigated a possible role of TFF3 during endochondral ossification in mice. They demonstrated that TFF3 is not present in areas of resting healthy growth plate ossification but can be visualized in areas of endochondral ossification. The localization of TFF3 was similar to that reported in OA cartilage suggesting similar roles for TFF3 during the two different processes [30].

OA is not only restricted to articular cartilage but affects the whole joint structures including also especially the synovial membrane (SM) which takes part in the etiology and the progression of
the disease [31]. Our here presented results reveal the presence of TFF3 on mRNA and protein level in SM of OA and RA affected knee joints and, in contrast to articular cartilage, also in healthy SM. This finding was confirmed by detecting TFF3 also on protein level and localizing studies in healthy human SM of differently aged healthy donors.

Completely unexpected, our data reveal expression of not only TFF3 but also TFF1 and TFF2 in synovial fluid (SF) of healthy individuals and patients suffering from OA and RA leading to the question of the source of TFF1 and TFF2. It seems obvious that the detected TFF3 is expressed and secreted into SF by SM. This is in accordance with the RT-PCR and immunohistochemistry results revealing expression of TFF3 in SM and suggesting an active secretion of TFF3 into SF. In addition, articular chondrocytes might be able to contribute to the relatively high TFF3 content in SF as they are able to produce TFF3 [19] whereas TFF1 and TFF2 are not produced in articular cartilage chondrocytes in both healthy articular joint structures and also OA or RA affected joint structures. One possible explanation for the presence of TFF1 and -2 in SF could be the fact that SF does not exclusively consist of proteins produced by SM or other joint structures. As an ultrafiltrate of blood plasma, it is primarily composed of proteins (including mucins) and small molecules such as glucose, urea and electrolytes that are derived from plasma in the blood vessels of the articular joint capsule [32, 33] and transported through the SM acting as a kind of selective filter [7]. This mechanism of a diffusion from blood plasma through SM into the joint cavity is also possible for TFF peptides and it would explain the small amount of TFF1 and -2 in comparison to TFF3 in SF as the ladder is additionally secreted into SF by SM and also articular chondrocytes. Moreover, it has been demonstrated for other body fluids such as for example saliva [34] or blood serum [35] that the concentration of TFF3 is regularly higher than that of TFF1 and -2.

Nevertheless, the possibility cannot be ruled out that the detection of TFF1 and -2 in SF samples might also be based on unspecific binding or cross reactive antibody reactions. The effect of heterophilic antibody reactions on the reliability of assays has been investigated in several studies [36]. Hampel et al. evaluated and discussed their effect in assays for chemokine and cytokine levels in OA and RA synovial fluid [37] and in particular detected false positive reactions for chemokines in the assay of RA synovial fluid samples. Furthermore, Samson et al. validated commercial assays for measurements of TFFs in serum [38]. They revealed acceptable results for TFF2- and TFF3-commercial assays but not for TFF1-assays which showed a poor precision and a narrow measuring range. An absolute reliable identification of the protein bands detected in western blot analysis is only possible by means of protein sequencing and will be a matter of further investigations.

Taking a closer look at the molecular structure of TFF peptides, there is, besides the six cysteine residues that form intramolecular disulfide bonds determining the TFF domain, an additional seventh cysteine at the C-terminal end of the amino acid chain that allows TFF1 and TFF3 to form intermolecular homo- or heterodimers [17, 39, 40]. We detected several distinct protein bands at different molecular weights. This is contrary to our expectations as we performed western blot under reducing conditions. We therefore expected only distinct bands at about 7 kDa (TFF1 and -3) and 12 kDa (TFF2) representing TFF monomers. The findings suggest a possible presence of non-reducible bounds between the TFF peptides and their binding partners or posttranslational modifications of the TFF peptides in SF. Several studies exist that indicate specific protein bands for TFF1, -2 and -3 heterodimers at higher molecular weights [41]. SF of OA and RA comprises a huge amount of different proteins that could be able to interact with TFF peptides performing, for example, disulfide bonds [37]. It is unclear so far which proteins are able to directly interact with the TFF peptides in SF leading to heterodimers or complexes. This will be subject in future studies as well as determining a possible function of these interactions in healthy synovial joint structures and in OA and RA.

Hypothetically one option could be the modification of the viscosity of SF. This effect has already been demonstrated for TFF peptides by Thim et al. in mucin solutions [13]. It is well known that TFF peptides and mucins are specifically co-expressed in mucin-producing epithelia especially of the gastrointestinal tract. Here, for example, TFF3 interacts with MUC5AC in human stomach and with MUC2 in the duodenum while TFF2 is co-produced with MUC6 in human stomach and duodenum [42]. TFF1 is also bound to MUC5AC in human gastric mucosa [43]. The exact molecular mechanisms
of these interactions, however, are still not understood. By now there are only few studies concerning
the expression of mucins in synovial tissue. Violin et al. detected an upregulated mucin expression,
particularly MUC3, and also the expression of MUC5AC in synovial tissues of OA and RA affected
human synovial joints in comparison to healthy tissues [44]. In contrast to Violin et al. a newer study
also found MUC1 to be present in synovial membrane cells as well as mononuclear cells in RA
synovial tissues, but not in OA, hypothesizing that mucins may also play a role in
immunoinflammatory reactions in the pathogenesis of RA [45]. Nevertheless, further investigations
are required to make a clear statement about possible functions of mucins in OA and RA synovial
fluid.

Our data reveal a downregulation of TFF3 between healthy and OA (statistically significant) as
well as RA (not statistically significant) SF samples and an upregulation of TFF3 between healthy and
OA (not statistically significant) SM samples. RA SF samples showed no significant changes. With
regard to articular cartilage chondrocytes the increased production of TFF3 in SM is in accordance
with the already mentioned results of Rösler et al. concerning the increased TFF3 expression in
articular cartilage under inflammatory conditions (OA and septic arthritis). Inflammation of SM
usually happens during the progress of OA [22] but there are also studies that indicate the existence
of SM inflammation also in earlier stages of OA as a possible factor in the pathogenesis. Benito et al.
detected, for example, a higher expression of TNFα and IL-1β as well as a higher cell infiltration and
vascularization in synovial tissue of patients with early OA compared to late OA [46]. This leads to
the hypothesis that synovial membrane inflammation might occur before the first signs of cartilage
degradation. The samples we used for real-time RT-PCR and ELISA were obtained from patients who
underwent total knee arthroplasty. This is usually performed in late stages of OA or RA meaning a
high level of cartilage degradation already present but not necessarily a high level of synovial
membrane inflammation. This could be one explanation for the differences of TFF3 seen in SM and
SF. However, all this is speculative and so far the different measurements are purely descriptive as
for example in other tissues chronic inflammation or trauma have been shown to be associated with
a downregulation of TFF3. Thus, Chaiyarit et al. detected a significant reduction of TFF3 expression
in oral mucosa of patients with chronic periodontitis compared to healthy mucosa [47] and Siber-
Hoogeboom et al. described significantly lower TFF3 saliva protein concentrations in cases of
rhonchopathy and obstructive sleep syndrome (OSA) compared to a healthy control group [28].

Although both diseases, OA and RA, show signs of synovial membrane inflammation, most
studies that compared synovial membrane inflammation in OA and RA found higher expression
levels of cytokines and a higher number of infiltrating immune cells in RA tissues [48]. Looking at
the results of Rösler et al., who detected an induction of TFF3 gene expression due to stimulation of
cultivated primary chondrocytes with TNFα and IL-1β [19], and considering the fact that TNFα and
IL-1β are well known as important mediators of inflammation [49, 50] it seems possible that this
mechanism is the reason for the significant higher level of TFF3 in RA synovial fluid.

Beside the observed differences of TFF3 we detected a highly significant upregulation of the
TFF2 protein concentration in SF from patients suffering from RA whereas in contrast nearly no
regulation was found in case of OA. As mentioned above, the composition of TFF peptides in SF
might also be influenced by the blood serum levels of the TFF peptides. Therefore, it would be
important to have knowledge about the human donors of SF whether these suffered from any
additional disease that could have had an influence on the serum levels. One example for this is
diabetes mellitus type 1. Barrera Roa et al. demonstrated that the TFF3 expression is regulated by
insulin and glucose [51]. Serum TFF3 levels are downregulated in serum of diabetes mellitus type 1
patients and presence of glucose and insulin results in elevated serum levels of TFF3. Similar effects
have been reported by Vestergaard et al., who detected elevated serum levels of TFF1 and -3 in
patients suffering from inflammatory bowel disease [52] and a study of Viby et al. found elevated
serum levels of TFF1, -2 and -3 in patients with COPD [53]. Furthermore, a higher serum level of TFF3
was detected in patients with chronic kidney disease, metastatic and secondary carcinoma and acute
gastroenteritis [54] as well as TFF2 serum concentrations were recently shown to increase in chronic
kidney disease [55]. Moreover, also a treatment of the patients with antiinflammatory medication
might have an effect on the expression of TFF peptides. Koitabashi et al. detected an increased TFF2 expression in the gastric cancer cell line MKN45 after incubation with indomethacin, a non-steroidal anti-inflammatory drug [56]. As also our RA patients received previously medication (we have not evaluated this) we can not exclude contributory effects. All these mechanisms could influence the levels of TFF1, -2 and -3 in SF and limit the findings of the present study. Nevertheless our findings can contribute to a better preparation and collection of sample data (medication, additional diseases) for example when analyzing other ultrafiltrates of blood plasma like urine, saliva or lacrimal fluid.

4. Materials and methods

4.1. Human tissues/fluids

All performed investigations followed the Declaration of Helsinki for research involving human tissue and with approval by the local ethics committee. Informed consent was obtained from each patient before surgery.

4.1.1. Synovial membrane

Healthy synovial membrane (SM) (n = 14) was obtained, with institutional review board approval, by the Department of Legal Medicine, Charité Berlin, Germany of male and female donors aged minimum 3 and maximum 92 years who were autopsied in cases of sudden and violent death. The samples were used for reverse transcriptase-polymerase chain reaction (RT-PCR), real-time RT-PCR, immunohistochemistry and ELISA.

SM of patients suffering from osteoarthritis (OA) (n = 10) or rheumatoid arthritis (RA) (n = 10) were obtained from male and female patients aged between 56-81 (OA) and 38-84 (RA) respectively who underwent total knee arthroplasty at the Department of Trauma Surgery, University Hospital Erlangen, Germany. These samples were used for conventional RT-PCR, real-time RT-PCR and ELISA.

4.1.2. Synovial fluid

Healthy human synovial fluid (SF) (n = 13) was obtained from donors, 4 female and 9 male, aged between 29-80 by the Department of Legal Medicine, Halle, Germany from autopsy cases of unnatural manner of death.

OA and RA SF (each n = 20) came from two different sources: ten OA samples (7 female and 3 male, aged 64–87) and ten RA samples (9 female and 1 male, aged 39–76) of SF were obtained from patients who were undergoing total knee arthroplasty at the Department of Trauma Surgery, University Hospital Erlangen, Germany. Another ten OA samples (6 female and 4 male, aged 65–84) and ten RA samples (6 female and 4 male, aged 57–77) were provided by the Department of Molecular Immunology, Friedrich Alexander University Erlangen-Nürnberg, Germany. The diagnosis of OA was based on clinical and radiographic evaluations according to standard criteria and the diagnosis of RA was made from patients fulfilling the American College of Rheumatology/European League Against Rheumatism classification criteria [27]. Each patient gave informed consent prior to surgery, and the institutional ethics committee approved the study (Ref.No. 3555; FAU Erlangen Nürnberg).

SF samples were used for Western blot analysis and Enzyme-linked Immunosorbent Assay (ELISA).

4.1.3. Human control tissue

A tissue sample of human stomach was obtained from a body donor (male, 83 years) donated to the Institute of Anatomy and Cell Biology, Martin Luther University Halle-Wittenberg, Germany who died by natural cause and did not suffer from any affection of the gastrointestinal tract.

4.2. RNA isolation and complementary DNA (cDNA) synthesis

Frozen samples of SM were homogenized with Speedmill Plus (Analytik Jena AG, Jena, Germany) and total RNA from healthy (n = 6), OA (n = 2) and RA (n = 2) samples of human SM was
4.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

cDNA of healthy (n = 6), OA (n = 2) and RA (n = 2) samples was amplified performing RT-PCR with specific primer pairs for TFF1, -2 and -3 as previously described by Hoogeboom [28]. To estimate the amount of amplified PCR product, a β-actin PCR was performed as loading control. Positive control (human stomach) and negative control (replacement of cDNA by RNA-free water) were included in the investigation.

4.4. Real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR)

The expression level of TFF3 in healthy (in total n = 12, each 2 samples were pooled to n = 6), OA (n = 5) and RA (n = 5) was analyzed after RNA isolation (as described above) by quantitative real-time RT-PCR (qRT-PCR) using Quant-Studio 12 K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) with TaqMan probes (5′ - Fam - CTGCTGCAAAACAGCTGCCC - Tamra - 3′) as fluorescent dye in the gene expression assay Verso 1-step RT-qPCR ROX Mix (# AB-4101/A; Thermo Fisher Scientific Inc., Waltham, MA, USA). Verso 1-step RT-qPCR ROX Mix is an assay that performs whole qRT-PCR (including cDNA-synthesis) in a single step assay. For detailed information and cycling protocol see product sheet. The relative amount of target mRNA of TFF3 was calculated and normalized to that of HPRT (hypoxanthine-guanine-phosphoribosyltransferase) mRNA (Gene Expression Assay HS03929096_g1; Life Technologies, Carlsbad, CA, USA) functioning as endogenous control, as HPRT has like TFF3 a low expression level. For quantitation the relative standard curve method was performed and the relative gene expression of TFF3 in healthy samples was afterwards compared to OA and RA sample groups. The used primers are listed in Error! Reference source not found.1.

Table 1. Primers used for RT-PCR and real-time RT-PCR.

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<th>Primers used for RT-PCR</th>
<th>Primers used for real-time RT-PCR</th>
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<tr>
<td>TFF3</td>
<td>TCCAGCTCGGCTGAGGAGTA</td>
</tr>
</tbody>
</table>

4.5. Western blot analysis

TFF1, -2 and -3 were analyzed by Western blot in healthy, OA and RA synovial fluid (each n = 2). The samples were diluted with PBS (phosphate buffered saline) and protein concentration was
spectroscopically measured by Bradford protein assay. Each sample (30 μg protein) was mixed with reducing sample buffer (RSB) containing 0.5 M dithiothreitol (DTT), 50% Glycerin, 0.05% Bromphenolblau, 20% β-Mercaptoethanol (β-ME) and boiled for 5 minutes. SDS-PAGE and Western blot analysis was performed as described by [29]. The membrane was incubated with primary antibodies to TFF1 (dilution 1:100), TFF2 (dilution 1:70) and TFF3 (dilution 1:400) at 4°C over night before applying the secondary antibodies (dilution 1:4000) conjugated to horseradish peroxidase for 2 h and detecting the bands by chemiluminescence using ECL Plus (Amersham Pharmacia, Uppsala, Sweden). Human stomach served as a positive control for all three peptides. The molecular weights of the detected protein bands were estimated referring to a standard protein marker (PageRuler Prestained Protein Ladder #26616; Thermo Scientific, Sweden).

### Table 2. Primary and secondary antibodies used for western blot (WB) and immunohistochemistry (IHC).

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Method</th>
<th>Specifity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>rabbit anti-TFF1</td>
<td>IHC</td>
<td>monoclonal</td>
<td>AJ1765a, Abgent</td>
</tr>
<tr>
<td>rabbit anti-TFF1</td>
<td>WB</td>
<td>polyclonal</td>
<td>orb100742, Biorbyt</td>
</tr>
<tr>
<td>goat anti-TFF2</td>
<td>IHC, WB</td>
<td>polyclonal</td>
<td>SP (P-19): sc-23558, Santa Cruz</td>
</tr>
<tr>
<td>mouse anti-TFF2</td>
<td>WB</td>
<td>monoclonal</td>
<td>H00007032-M01, Abnova</td>
</tr>
<tr>
<td>rabbit anti-TFF3</td>
<td>IHC</td>
<td>polyclonal</td>
<td>ABIN 669786, Bioss Inc.</td>
</tr>
<tr>
<td>rabbit anti-h-TFF3</td>
<td>WB</td>
<td>polyclonal</td>
<td>provided by Prof. W. Hoffmann, Magdeburg</td>
</tr>
<tr>
<td>rabbit anti-Actin</td>
<td>WB</td>
<td>polyclonal</td>
<td>Actin (H-300): sc-10731, Santa Cruz</td>
</tr>
<tr>
<td>rabbit anti-alpha-1-antitrypsin</td>
<td>WB</td>
<td>polyclonal</td>
<td>A 0012, Dako</td>
</tr>
<tr>
<td>goat anti-rabbit</td>
<td>IHC</td>
<td>polyclonal</td>
<td>BA-1000, Vector Laboratories Inc.</td>
</tr>
<tr>
<td>rabbit anti-goat</td>
<td>IHC</td>
<td>polyclonal</td>
<td>E 0466, Dako</td>
</tr>
<tr>
<td>goat anti-rabbit</td>
<td>WB</td>
<td>polyclonal</td>
<td>P 0448, Dako</td>
</tr>
<tr>
<td>goat anti-mouse</td>
<td>WB</td>
<td>polyclonal</td>
<td>IgG-HRP: sc-2005, Santa Cruz</td>
</tr>
<tr>
<td>donkey anti-goat</td>
<td>WB</td>
<td>polyclonal</td>
<td>IgG-HRP: sc-2020, Santa Cruz</td>
</tr>
</tbody>
</table>

### 4.6. Immunohistochemistry

Samples of human SM of a 22 (male), a 48 (male) and an 83 (female) year old healthy donor (fixed in 4 % formaldehyde) were paraffin-embedded and immunohistochemistry was performed on 5 μm sections of the tissue with antibodies to TFF1, -2 and -3. For antigen retrieval, the sections were cooked in citrate buffer (pH = 6.0) for 5 minutes. To inhibit nonspecific binding the sections were incubated with normal serum for 20 minutes using normal goat serum for TFF1 and -3 and normal rabbit serum for TFF2 (Dako, Glostrup, Denmark, dilution: 1:20 with TBST). Sections were incubated with the primary antibodies (dilution: 1:100 (TFF1, -2), 1:300 (TFF3) with TBST) in a humidified box over night at 4°C and with the biotinylated secondary antibodies (dilution 1:200 with TBST) for 1 h. For visualization of the antibody reaction, AEC+High Sensitivity Substrate Chromogen (K3469, Dako, Glostrup, Denmark) was used for about 2-3 minutes depending on the used antibody. The results were examined by using light microscopy (Biorevo microscope BZ-9000, Keyence, Osaka, Japan). Both, positive (tissue slides of stomach) and negative (incubating slides only with secondary antibody) controls were included in immunohistochemistry. The used primary and secondary antibodies are listed in Error! Reference source not found.2.

### 4.7. Enzyme-Linked Immunosorbent Assay (ELISA)

For quantitative detection of TFF1, -2 and -3 in healthy (n = 13), OA (n = 20) and RA (n = 20) human synovial fluid and of TFF3 in healthy (n = 10), OA (n = 10) and RA (n = 10) human synovial membrane the following ELISA-Kits from USCN Life Science Inc., Wuhan, PRC were used:
5. Conclusion

In summary, our results demonstrate the presence of trefoil factor family peptide 3 (TFF3) in synovial membrane and unexpectedly show that synovial fluid contains all three TFF peptides that with high probability originate from blood serum. Significant changes in the TFF peptide concentration of synovial fluid between healthy individuals and patients suffering from OA and RA suggest a possible role in the pathogenesis of both diseases. However, to get further insights into possible functions of TFF peptides in healthy individuals and cases of OA or RA we need future studies with more background information about the patients under investigation.

Author Contributions: F.P. initiated the project. J.P. investigated the localization and detection of TFF in the synovial membrane. M.S. and J.P. performed the ELISA quantification of TFF. P.K., K.K., S.S., M.T., S.E. D.S. and H.C. provided numerous samples for the experiments. J.P and F.G. performed the statistical analysis. F.P. supervised the project. J.P. and F.P. wrote the manuscript with input from the other authors.

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Conflicts of interest: The authors declare no conflict of interest.

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