UiT
THE ARCTIC UNIVERSITY OF NORWAY

Department of Clinical Dentistry
Faculty of Health Sciences

The difference in salivary biomarkers comparing healthy children and children with juvenile idiopathic arthritis

Karoline Rebne, Ingrid Rørbakken and Cristell-Mari Hissink
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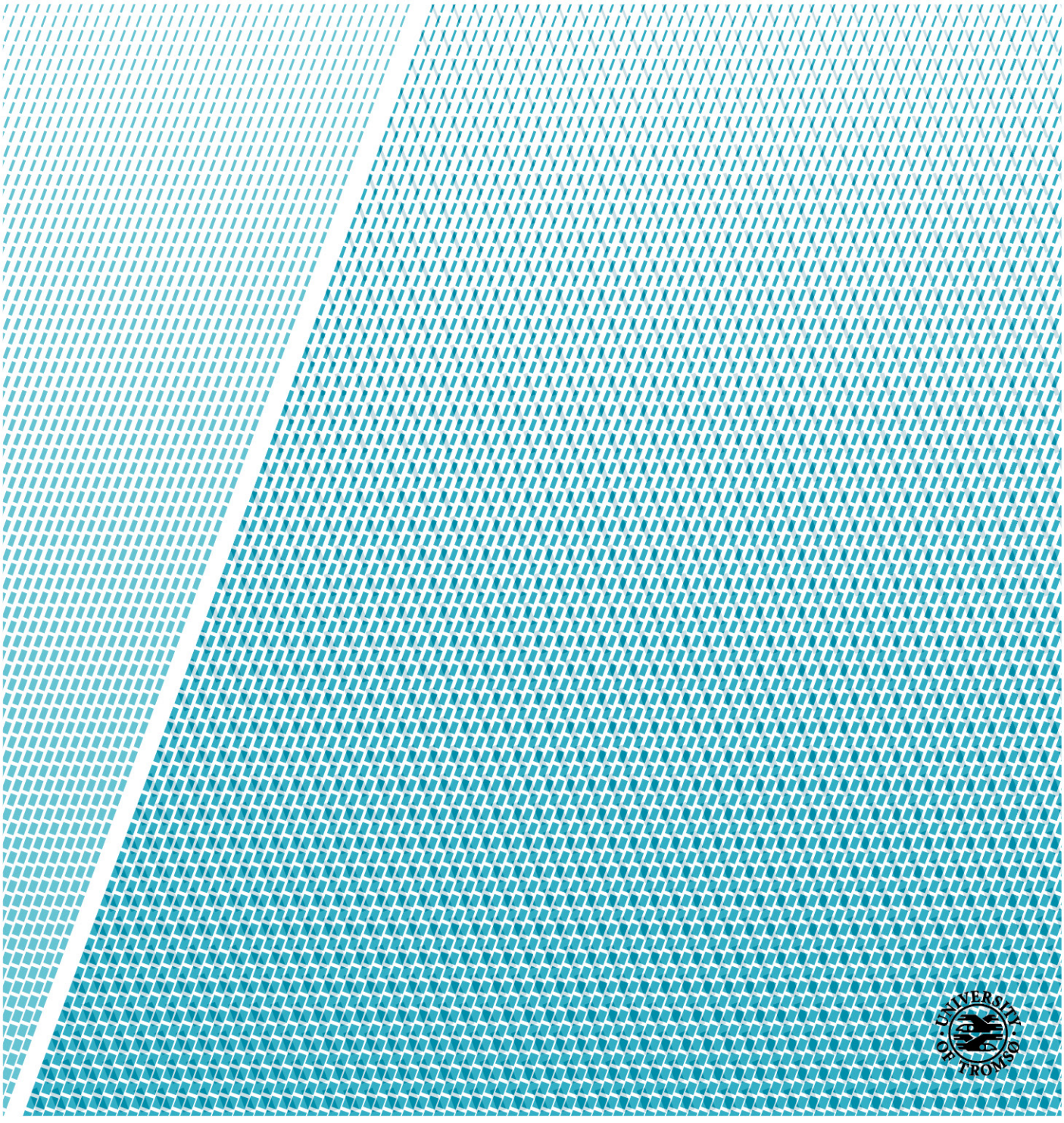
Supervisors: Mohammed Al-Haroni
Marwan Mohammed



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Abstract

Aims:

Children with juvenile idiopathic arthritis (JIA) have a chronic inflammatory condition often with a fluctuating disease course. Could increased levels of bacteria and inflammatory markers be a marker of disease onset and disease activity, or is it simply a consequence of the disease? Our aim with the study is to investigate inflammatory markers and two selected bacteria found in saliva in children with JIA compared to healthy children.

Materials and method:

Salivary samples were collected under NorJIA study, a larger Norwegian prospective multicenter cohort study on JIA. The saliva samples were used to investigate the levels of inflammatory markers and levels of *S. mutans* and *P. gingivalis*. The inflammatory markers were measured using BioPlex technology which gives us the opportunity to measure several biomolecules in the same samples simultaneously. Extracted DNA from the saliva samples were amplified and quantified by using a droplet digital PCR (ddPCR). The Mann-Whitney and the Kruskal-Wallis test was used for not normally distributed variables, and for cytokines the p-value set to > 0,01 to adjust for multiple variables.

Results:

In ddPCR quantification in saliva *S. mutans* was significantly higher in the JIA group compared to the healthy control group. For the cytokine analyses IL-2, IL-9, IL-17, basic FGF and G-CSF were significantly higher in the healthy control group compared to JIA. RANTES had significantly higher levels in the JIA group compared to the healthy control group.

Conclusion:

In this study, we found support for our hypothesis that children with JIA have a different concentration of inflammatory markers and a higher amount of *S. mutans* in their saliva compared to children without JIA. Further research is needed to reveal the association between higher amount of *S. mutans* and the different levels of some proinflammatory cytokines in saliva in JIA patients.

1 Introduction

1.1 Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is a diverse group of diseases of unknown origin, all defined by arthritis with onset before age 16 years (1). JIA is classified as an autoimmune disease, and is the most common childhood chronic rheumatic disease (2). It is also an important cause of morbidity in the developed world (3). In high-income countries the disease has an incidence of 2-20 per 100 000 population yearly, and a prevalence equivalent to 16-150 cases per 100 000 population (1).

1.1.1 JIA and oral health

The effect of JIA on oral health is poorly documented, and the number of studies and patients included is often small. However, some former studies have reported increased amounts of caries and periodontitis in patients with JIA. Upper limb involvement in the JIA may lead to severe functional disability and poor fine motor movements. This may increase the difficulty of toothbrushing and flossing, and thus plaque removal. In 40-70% of the cases of JIA, the temporomandibular joint (TMJ) is involved (4). This can cause destructive changes of the TMJ's form and function (5). If the TMJ is involved in the JIA it can cause restriction in mouth opening, and may also interfere with plaque removal (4).

Saliva consist of both organic and inorganic components and is crucial to maintain good oral health. Some of saliva functions include dental remineralisation, antimicrobial activity, buffering capacity and regeneration of mucosa (6).

Hence, saliva is important to prevent the development of both caries and periodontal disease. Previous studies reveal various results regarding the quantity and quality of saliva in JIA patients. Some studies showed altered composition and/or reduced salivary flow, other studies showed no significant difference at all (7).

1.2 Oral bacteria

Studies has estimated and found almost 700 different predominant bacterial species in the oral cavity. There is a clear site-specificity existing, even though there is a certain overlap between these species and their location. The oral microbiome has important implications for both health and disease, and is easily accessible compared to other body sites (8). The most predominant species in a healthy oral environment is the streptococcus. The saliva

composition varies between individuals, but other abundant species are Haemophilus, Neisseria, Prevotella, Veillonella and Rothia (9).

Research suggests that bacterial flora in the gut is connected to the development and function of the immune system. Evidence also shows that children with JIA have an altered intestinal microflora. In addition to the bacteria in the gut, scientists have showed interest in exploring microbiomes also from the respiratory tract and oral cavity, and believe they may play a role (10).

1.2.1 Streptococcus mutans

This facultative anaerobic bacterium is a natural part of the oral microbiome, but are considered to be the most cariogenic bacteria (11). *Streptococcus mutans* is capable of adhesion to solid surfaces, colonize the oral cavity and form bacterial biofilm (12). The microorganism also possesses several sugar transport systems, and even under extreme environmental conditions, such as low pH, it maintain the ability to grow and metabolize sugar (11). The ability to adhere to solid surfaces and the sugar transport systems are caries-promoting, and hence the microorganism is one of the many etiological factors of dental caries (12).

1.2.2 Porphyromonas gingivalis

This gram-negative anaerobic bacterium is a part of the oral microbiome, but it is also involved in the pathogenesis of periodontitis and can become severely destructive. The bacteria disturbs the epithelial cells and initiates an inflammatory and immune process that leads to destruction of the tissues surrounding and supporting the teeth. *Porphyromonas gingivalis* possesses several virulence factors that allows it to locally invade the periodontal tissues and avoid the host defence mechanisms (13).

1.3 Diagnostics and biomarkers

Today there is no conclusive laboratory tests for the diagnosis of JIA available, and the diagnostics may therefore be time consuming and comprehensive for both the children and the clinicians. It has with time been shown that an increased understanding of the disease has contributed to earlier diagnosis, which is essential to prevent long term damage to joints (14).

The diagnosis is today made by a good anamnesis, physical examination, clinical features and different laboratory tests, including blood examination, inflammatory markers and immune markers (14). The clinical features are strongly related to the subtype of JIA, and differs in among others the age of onset, the joints involved, uveitis, and the presence of different biomarkers (14).

Measurement of inflammatory markers is used to detect acute inflammatory markers that might indicate specific diseases (15). While blood is generally regarded as the best body fluid for evaluation of systemic processes, substitution of saliva samples for blood would be less invasive and more convenient (16).
As mentioned, there is no specific lab test for JIA today, but there are certain markers in the blood that points in the direction of the diagnosis. Among these are modestly elevated CRP and erythrocyte sedimentation levels, thrombocytopenia, and a microcytic anemia (17).

1.4 Cytokines

Research studies have reported a linkage between JIA and several specific cytokines in the serum and synovial fluid. These include IL-6, TNF-α, IL-10 and MIF (macrophage migration inhibitory factor). In addition, the inflammatory process connected to the disease produces many mediators, and this leads to higher levels of pro-inflammatory cytokines. By monitoring these changes, it can be possible to use these measurements as therapeutic or prognostic biomarkers for JIA (18).

When active JIA disease is present, the plasma cytokine concentration is proved to be increased 2-35 times. In the synovial fluid, this increase is even more remarkable. The following cytokines is then commonly found in higher levels: IL-6, IL-5, CCL2, CCL3, CXCL9, CXCL10 (18).

Even though there are evidences supporting the increase in specific cytokines in the plasma and synovial fluid of JIA children, these biomarkers levels are not diagnostic for JIA. Some of the drawbacks include difficulties in gathering all samples at the exact site of inflammation, and the availability of some of the cytokines is under neuroendocrine control, which can vary throughout the day and night. In addition, we must remember that the disease has a very

complex immune process, and that the cytokines never can be anything other than a reflection of this complexity (18).

Throughout our research and lab work, we analysed our samples for 27 different cytokines.

Table 1: Cytokines.

IL-1β	IL-10	IFN-γ
IL-1ra	IL-12p70	IP-10
IL-2	IL-13	MCP-1
IL-4	IL-15	MIP-1α
IL-5	IL-17A	MIP-1β
IL-6	Eotaxin	PDGF-BB
IL-7	Basic FGF	RANTES
IL-8	G-CSF	TNF-α
IL-9	GM-CSF	VEGF

1.5 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a technique for quantifying and detecting molecular targets such as proteins. ELISAs are often performed in 96-well polystyrene plates that binds and immobilize the antigen of interest, for example, inflammatory marker, and makes it possible to separate bound from non-bound material. The immobilization of the antigen can be achieved by direct adsorption to the assay plate or indirectly by attaching capture antibodies to the plate. An assay format called sandwich assay involves the antigen of interest being attached between the captured antibody and the detection antibody. This assay provides an accurate and sensitive detection of the antigen. ELISAs has many desirable features, but the method is limited by its ability to only measure one single antigen in each sample.

1.6 Bio-plex

Bioplex arrays have been developed from the ELISA technology. This gives the new technique the opportunity to measure several biomolecules in the same sample at once. Other advantages of using bioplex over ELISA are that less sample volume is needed, it is more efficient time and cost wise, and gives higher sensitivity and specificity results. Bioplex is frequently used in research dealing with inflammation, cancer markers, autoimmune diseases, diabetes etc (19).

The bead-based multiplex technology enables a multiplex analysis using different coloured beads with different antibody pairs, and makes detection of several targets in one biological liquid sample possible. The previous steps are similar to the ELISA test (19).

1.7 Extraction of DNA

QIAcube (Qiagen) is a semi-automated system for nucleic acid extraction. The machine uses a chemical enzymatic lysis and silica spin-column binding isolation method, and has a batch capacity of 12 samples at the time (20).

1.8 Droplet digital polymerase chain reaction (ddPCR)

The ddPCR is a new technology that allows thousands of amplification reactions to arise at the same time through utilization of emulsion droplets. Which results in a higher probability of amplifications of rare target sequences. ddPCR have a better reproducibility and sensitivity than other PCR techniques (21).

2 Hypothesis and aims

Our aim in the master project is to investigate the inflammatory markers and two selected oral bacteria found in saliva in children with JIA compared to healthy children.

Our hypothesis is that children with JIA have a different concentration of inflammatory markers, and a higher amount of *S. mutans* and *P. gingivalis* in their saliva compared to children without JIA.

3 Materials and methods

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK Nord; approval no. 2015/318).

3.1 Saliva samples

For our master project, the saliva samples was obtained from NORJIA. We received 94 collected saliva samples, where 59 of them were samples from children with JIA, and 34 from healthy children without arthritis. In our research, we also included 9 samples of synovial fluid collected from the TMJ of children diagnosed with the disease.

The inclusion criteria for the JIA group were children from the Norwegian participating centers, diagnosed with JIA, aged <18 and with arthritis activity in one or both of the temporomandibular joints.

3.1.1 The Laboratory work

1. Extract DNA from the saliva samples using a QIACube and QIAamp DNA minikit. The ddPCR technique was used to search for the DNA of interest.
2. Analysing which cytokines are present in the saliva samples and their levels using the Bio-Plex method.

3.2 Extraction of DNA and ddPCR

3.2.1 Extraction of DNA and DNA concentration measurement

The DNA was extracted from the saliva samples using the QIACube technology and QIAamp DNA Mini Kit (Qiagen). This is an advanced process for creating spin columns, to obtain a seamless integration of automated sample preparations. Throughout the process, there is no need for purification and human chemical adjustments. This leads to a faster process that give rapid results.

In the first step, 500 to 700 µl of saliva were transferred to tubes and diluted with an equal amount of Phosphate Buffered Saline mix (PBS). Then these tubes were centrifuged for 5/10 minutes at 14800 rpm to pellet the bacteria. Next, the supernatant in each tube was removed. The bacterial pellet was again mixed with 160 µl PBS and 20 µl metapolyzyme, and further incubated for 4-5 hours in a warming block, at 35 °*C*.

Further, to confirm the success of the extraction process, agarose gel electrophoresis, was used, and then visualised with an UV-trans-illuminator called ChemiDoc Touch Imaging System (Bio-rad). To measure the concentration of the extracted DNA, a Qubit Fluorometric device (ThermoFisher Scientific) was used according to the manufacturer´s protocol.

3.2.2 Droplet digital Polymerase chain reaction

The saliva samples contained both DNA from the individual and from the oral bacteria. In this project, it is particularly *S. mutans* and *P. gingivalis* that are of interest. To check for their

presence in the saliva samples using a complex new PCR technology, called ddPCR (droplet digital polymerase chain reaction), which detects bacteria through their DNA (22).

Firstly, the saliva samples were diluted to achieve a DNA concentration of 150 pg/µl in each sample. Then the master mix with a total volume of 19 µl was prepared, containing 1 µl primer, 1 µl enzyme, 7 µl H_2O and 10 µl polymerase (supermix). 19 µl master mix was added to separate tubes, before adding 1 µl DNA (50-100 bp), giving a total volume of 20 µl.

Further, the prepared samples and droplet generator oil were transferred to a DG8 cartridge and placed in the droplet generator. After the generation of droplets, a total of 40 µl of droplets were transferred to the 96-well PCR plate, before sealing it with a foil at 180°*C* using a plate sealer (PX1TM PCR Plate Sealer, Bio-Rad*)*.

Then the PCR amplification was carried out within all of the droplets, using a Thermal Cycler (C1000 Touch ™ Bio-Rad). Firstly, the enzymes were heated and activated at 95 °C for 10 minutes. Then the amplification process follows, and is characterised by a cycle of denaturation for 30 seconds at 95°C, and annealing and extension for 1 minute at 56 °C. This cycle was repeated and performed 40 times, and in the end, a 10-minute-long deactivation of the enzyme at 98 °C was carried out. Lastly the plate with the droplets was transferred to droplet reader (Droplet Reader QX200 ™ BioRad), for analysis of each single droplet. The data obtained from this step was sent to a software (QuantaSoftTM), where the droplets were further analysed.

Table 2: Sequence of primers and probes used in the study

Test species	Sequence 5'-3'	Target gene	Amplicon size
P. gingivalis	F- primer: acgaatcaaaggtggctaagtt R- primer: atcagccgaaaatgcgacta Probe: ccgtaatggtttataatggagaacagcagg	Fimbrialprotein (fimA)	84 bp
S. mutans	F- primer:ggtaacctgcctattagc R- primer: atacaacgcaggtccatc Probe: gggggataactattggaaacgatagc	*AMY*	84 bp

3.3 Cytokine analysis

3.3.1 Initial preparation

Two hundred fifty microliter of saliva and synovial samples were centrifuged using MicroCL 21R Microcentrifuge (Thermo Fisher Scientific) for 5 minutes at 7000 rpm to pellet the

bacteria and debris. Then 50 µl of the supernatant was transferred into new tubes. This was stored at -80 °C until further analysis. To measure the cytokine concentration in the samples, the Bio-plex Pro™ Human Cytokine Standard 27-Plex kit was used according to the manufacturer's instructions. 1X Wash buffer, 1X assay buffer, 1X detection antibody and 1X beads solution were prepared. Then a fourfold standard dilution series (standard 1 to standard 9) were prepared, where the highest concentration of cytokine is standard 1. The concentration is shown in Table 3. Universal assay buffer was used as a blank.

Table 3: Bio-Plex Pro ™ Human Cytokine Standard 27-Plex, Group 1

Analyte	Standard PTM Setting (CAL2 Low RPI Target)	Analyte	Standard PTM Setting (CAL2 Low RPI Target)	Analyte	Standard PTM Setting (CAL2 Low RPI Target)
IL-1β (39)	1,017	IL-10 (56)	27,194	IFN-γ (21)	7,250
IL-1ra (25)	76,885	IL-12p70 (75)	19,999	IP-10 (48)	48,834
IL-2 (38)	18,719	IL-13 (51)	5,017	MCP-1 (53)	7,423
IL-4 (52)	1,585	IL-15 (73)	141,831	MIP-1α (55)	754
IL-5 (33)	60,963	IL-17A (76)	27,627	MIP-1β (18)	6,901
IL-6 (19)	8,348	Eotaxin (43)	1,717	PDGF-BB (47)	67,187
IL-7 (74)	37,661	Basic FGF (44)	63,088	RANTES (37)	51,984
IL-8 (54)	18,569	G-CSF (57)	109,119	TNF-α (36)	53,313
IL-9 (77)	40,176	GM-CSF (34)	6,760	VEGF (45)	178,228

3.3.2 Setting the cytokine assay

To analyse the saliva samples a 96-well assay plate and the Bio-Plex Pro™ Human Cytokine Standard 27-plex kit were used. This is a kit based on different coloured magnetic beads with different antibody pairs, which makes detection of several targets in one biological liquid sample possible.

Fifty µl of the diluted 1X beads were added to each well of the assay plate using a multiple pipette. The 96-well assay plate was steadily inserted into a Hand-Held magnetic plate washer before the liquid was removed. Then the plate was properly washed by adding 100 µl Wash Buffer 1X to each well. The washing was done twice, and the Wash Buffer was discarded both times. The samples, standards and blanks were vortexed before adding 50 µl to dedicated wells. Then the assay plate was covered with sealing tape and incubated on a shaker at 850 rpm at room temperature for 30 minutes.

After incubation, the plate assay was washed three times with 100 µl Wash buffer before adding 25 µl diluted 1X detection antibodies to each well. The plate was again sealed with sealing tape and incubated on a shaker at 850 rpm at room temperature for 30 minutes. After incubation the assay plate was washed three times with 100 µl wash buffer. Then 50 µl 1X Streptavidin-Phycoerytrin (SA-PE) was added to each well, before covering the plate with sealing tape and incubating on shaker at 850 rpm at room temperature for 10 minutes. The plate was then washed three times with 100 µl Wash buffer. Finally, the beads were resuspended in 125 µl assay buffer. The plate was covered with sealing tape and shaken at 850 rpm for 30 seconds. The sealing tape was removed, and the plate was read using low PMT, RP1 setting for the Bio-plex 200.

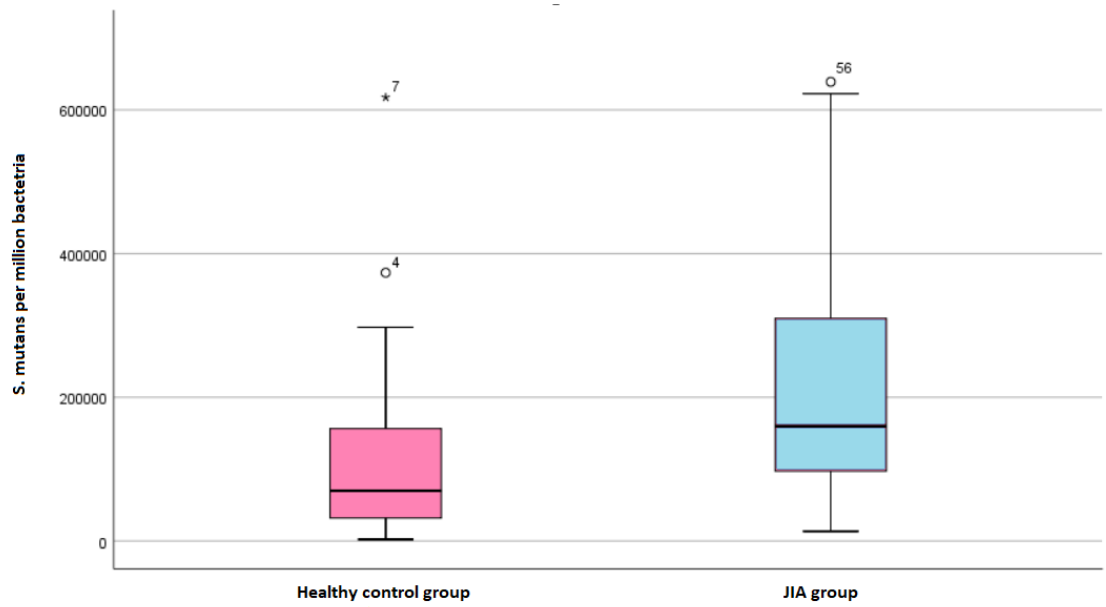
4 Results

4.1 ddPCR

The data were not normally distributed, therefore a Mann-Whitney test was used to analyse the results of the ddPCR. The difference between the JIA group and healthy control group were only tested for *S. mutans*, because the data for *P. gingivalis* was almost zero in most of the cases. From the results, it emerges that the null hypothesis has to be rejected. Hence, the JIA group has higher levels of *S. mutans* compared with the healthy control group (Figure 1).

As shown in appendix 1, in the JIA group the minimum level of *S. mutans* per million bacteria was 13498 bacteria and the maximum was 639037 bacteria, the mean was 209361. In the healthy control group the minimum level of *S. mutans* per million bacteria was 2275 bacteria and the maximum was 617414 bacteria. The mean was 117752.

Figure 1. Observed values of S. mutans per million bacteria in the two groups



4.2 Measurement of cytokines

A Kruskal-Wallis test was used for testing differences between the three groups; JIA, JIA with TMJ involvement and the healthy control group. The p-value was set to 0,01 because of the amount of analysis done simultaneously.

After taking the p-value into consideration, six cytokines had significantly different levels in JIA in the comparison to the HC group. Of these IL-2, IL-9, IL-17, basic FGF and G-CSF were present in higher amounts in the healthy control group. The only cytokine that gave significant results for the comparison of the JIA-TMJ group and the HC samples were G-CSF, with much higher levels in the healthy control group. RANTES, the final cytokine with a significant p-value, was the only one found in higher levels in the JIA saliva compared to the healthy controls.

4.3 Hypotheses testing

Our hypothesis ‘’children with JIA have a different concentration of inflammatory markers, and a higher amount of *S. mutans* and *P. gingivalis* in their saliva compared to children without JIA’’, was tested by using a Kruskal-Wallis test and Mann-Whitney test respectively. There are six inflammatory markers that differed significantly between the two groups, as well as significantly higher values of *S. mutans* in the JIA-group, hence the null hypothesis must be rejected.

5 Discussion

5.1 Methodical consideration

As previously mentioned, the laboratory work has consisted of complex and detailed processes that demand a high level of accuracy. The research has been carried out by three relatively inexperienced students, which might have influenced the preciseness of the results. It is nevertheless important to emphasize that the students have been under careful supervision throughout the entire laboratory work.

5.2 ddPCR

As previously mentioned, the effect of JIA on oral health is poorly documented. Some studies, however, have reported an increased amount of caries and periodontitis in patients with JIA (4). The fact that this study showed that the JIA group had higher levels of *S. mutans* as assessed by ddPCR than the healthy control group, supports these results. Nevertheless, it is important to stress that we have no information regarding the participant’s dental status, oral hygiene habits and other factors that might influence the bacterial amount.

The children with JIA might also have symptoms in the joints of the hands and fingers, and this can cause problems with a thorough brushing technique, and will further lead to plaque deposits, increased caries risk and higher amounts of *S. mutans*. If their temporomandibular joint is affected, this may lead to rediced mouth opening and trismus, which in turn can lead to the same as mentioned above.

The results of the *P. gingivalis* analysis pointed towards very low levels of the bacteria in our samples. This most probably correlates with the fact that periodontitis is rather infrequent and rare in children (23), and the risk increases with age (24).

5.3 Cytokines significantly higher in healthy control group

5.3.1 Interleukin 2

Interleukin-2 (IL-2) is mainly produced by CD4 lymphocytes and is an important factor in controlling the immune response. The production of IL-2 is dependent on two types of signals: 1) The binding of the T cell receptor (TCR) to the antigen/major histocompatibility complex (MHC), and 2) The binding of T cell surface molecules to B7-1 and B7-2 on the surface of antigen presenting cells. If none of the signals is present, the production of IL-2 will not occur (25). Studies done in IL-2 knockout mice have acknowledged that one of IL-2 most crucial activities is to downregulate responses in order to prevent autoimmunity. This correlates with the fact that healthy control group had significantly higher concentration of IL-2 than the JIA group (26).

5.3.2 Interleukin 9

The production of interleukin-9 (IL-9) is a complex matter and recent studies indicate that it has multiple cellular sources (27). Various signalling pathways and other cytokines are able to regulate the IL-9 production, whereby IL-2 is one of the promoters (28). IL-9 has for a long time been a cytokine of interest due to its involvement in the immune-pathogenesis of different inflammatory diseases, hereby asthma and allergic rhinitis, which both show increased levels of IL-9 in serum.

Increased expression of IL-9 has also been observed in the synovial tissue of people with rheumatoid arthritis (RA), and there are several factors indicating its pathogenic role in RA. However, the fact that type 2 innate lymphoid cells (ILC2s), which produce IL-9, play a role in the resolution of chronic inflammation indicates the opposite. Thus, it appears that IL-9 plays a role in both pathogenesis, as well as the resolution of chronic inflammation. The dominating effect depends on the cellular source and microenvironment. Some studies also indicate that IL-9 has a role in inducing immune tolerance by recruiting and activating mast cells, which is crucial for inducing tolerance (27).

It would have been interesting to analyse the synovial fluid of the participants of this trial, to see if the IL-9 levels would differ dramatically from the saliva samples. As previously mentioned, IL-9 has been found in high concentrations in the synovial tissues of people with RA, which might indicate that this also could be the case of the synovial fluid of the mandibular joint.

5.3.3 Interleukin 17

T-cells produce interleukin-17 (IL-17), which among other things stimulates stromal cell elements to release cytokines including G-CSF, IL-6 and IL-8 (29). The pro-inflammatory qualities of the cytokine are important for its host protective capacity, while rampant IL-17 signalling is related to autoimmune disease and immunopathology. Studies have shown that people with IL-17 defects are particularly susceptible to chronic mucocutaneous candidiasis, something that might indicate that IL-17 deficiencies can cause other pathology as well(30).

5.3.4 Basic-FGF

This cytokine and potent growth factor is of important value in the proliferation of fibroblast and endothelial cells. This can explain why growing young individuals have a significantly increased proliferative activity and high levels of B-FGF in their saliva, compared to older individuals. It is also released in wounded tissues, and wounds appear during exfoliation and tooth exchange throughout a 15-year period in young individuals. Basic FGF seems to be age dependent and decreasing with age, and a 185-sample study from Finland concludes with a mean B-FGF-value of young healthy individuals (aged 4-19) equal to 0.72 pg/mL.

Basic FGF has been linked to being involved in the maintenance of mucosal health in the entire upper digestive tract. Studies have also shown increased concentration of the cytokine in smokers compared to non-smokers (31).

Chronic inflammation is known to interfere with growth. Children with JIA may have growth disturbances and consequently might have lower levels of growth factor. This might explain why the healthy children in our study have a higher amount of basic-FGF in their saliva, compared to the children with JIA (31).

5.3.5 Granulocyte colony stimulating factor

Granulocyte colony stimulating factor (G-CSF), which is a haematopoietic growth factor, is mostly produced by haematopoietic cells and lymphocytes (32). Studies have shown that serum concentration of G-CSF in healthy individuals are non-detectable levels in over 90% of the cases, but increase dramatically during an acute bacterial infection. Thus, measurable plasma concentrations of the growth factor is one of the features of the inflammatory response

during infections. Nevertheless, the same study discovered low levels of G-CSF in patients with frequent infections. This might suggest that recurrent infections reduce the capacity of G-CSF production (32).

5.4 Cytokines significantly higher in JIA group

5.4.1 RANTES/CCL5

As mentioned in the results section, RANTES, "Regulated upon activation normal T-cell expressed and secreted" was the only cytokine measured in higher amounts in the JIA samples compared to the healthy controls. This chemokine is a powerful leukocyte activator, and induce the migration of classical lymphoid cells to sites of inflammation by binding to receptors in the GCPR family. RANTES is regarded as a particular feature of inflammation, and is produced by CD8 T cells, fibroblasts, platelets and epithelial cells. Elevated levels of RANTES has been linked to many different inflammatory disorders, and one of them is arthritis. This discovery points to a positive correlation between elevated saliva levels of the cytokine RANTES and JIA (33).

5.5 Difference between saliva and serum

Inflammatory markers may be measured in various biological specimens including saliva and synovial fluid, but blood is the standard practice when examining cytokines in the context of disease and clinical diagnosis (34). Experience shows that there are few studies comparing cytokine levels in saliva, synovial fluid and serum. Thus, it is difficult to determine the reliability and the significance of the cytokine levels in saliva

5.6 Fluctuations in inflammatory markers

When analysing the levels of cytokines in children's saliva, it is important to understand that there are various factors that might have an impact on these levels. The patient's age is of significance for the immune system and response. Hence, difference in age might lead to differences in the cytokine concentrations (35). In our study the children with JIA and healthy controls were matched according to age and gender.

It is well known that obesity leads to chronic systemic inflammation due to immune responses in adipose tissue. Studies have shown that obesity is associated with significantly elevated levels of several cytokines in serum. The cytokine production may, however, be modulated through physical activity (36).

Despite increasing amounts of studies, it is unclear if inflammatory markers in saliva reliably change in response to acute stress. However, studies have shown an increase in blood-based inflammatory markers when exposed to acute psychological stress, and this may be the case for these children.

Other than the fact that the healthy control group does not have JIA, we have no information regarding their general health condition. Therefore, we cannot exclude that they have other conditions, which might have affected the cytokine concentration in the saliva samples (37).

6 Conclusion

The current study attempted to investigate inflammatory markers and two selected oral bacteria in saliva of children with JIA compared to healthy children. We found an interesting link between children with JIA and significantly higher levels of *S. mutans*. With this in mind, it would have been interesting to see if they in fact do have poorer oral health and more caries compared to healthy children. Therefore, future follow up studies should address oral health in addition to saliva analysis.

As mentioned earlier, the inflammatory markers are a complex matter. This study has not given any results that are of diagnostic value. However, the fact that RANTES, which is a cytokine linked to many inflammatory diseases, was present in significantly higher levels in the JIA samples, is a very interesting discovery. It will require further research of the cytokine interplay to reveal correlations between this and other cytokines and the disease onset and activity, and we suggest more attention on this area in the future.

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Appendix 1 – *S. mutans* per million bacteria in the saliva of the study subjects

Health 1	2275
Health 2	17465
Health 3	119958
Health 4	373358
Health 5	216216
Health 6	63724
Health 7	617414
Health 8	96410
Health 9	205462
Health 10	45083
Health 11	295552
Health 12	9340
Health 13	40029
Health 14	297468
Health 15	31879
Health 16	236473
Health 17	7197
Health 18	39447
Health 19	51878
Health 20	76203
Health 21	80684
Health 22	200000
Health 23	44491
Health 24	150360
Health 25	14392
Health 26	24628
Health 27	21292
Health 28	23247
Health 29	34048
Health 30	61409
Health 31	141892
Health 32	156455
Health 33	109439
Health 34	98392
JIA 1	321003
JIA 2	290536
JIA 3	107731
JIA 4	117571
JIA 5	274586
JIA 6	79361
JIA 7	348266
JIA 8	67634
JIA 9	338390
JIA 10	292135
JIA 11	364629
JIA 12	18155
JIA 13	118008
JIA 14	103663
JIA 15	495502
JIA 16	123829
JIA 17	496781
JIA 18	257406
JIA 19	261339
JIA 20	488580
JIA 21	372270
JIA 22	639037
JIA 23	622496
JIA 24	107060
JIA 25	105080
JIA 26	290040
JIA 27	78251
JIA 28	184013
JIA 29	180043
JIA 30	158183
JIA 31	30350
JIA 32	150282
JIA 33	309764
JIA 34	333333
JIA 35	468750
JIA 36	498789
JIA 37	175630
JIA 38	205955
JIA 39	77224
JIA 40	329025
JIA 41	113505
JIA 42	214924
JIA 43	280811
JIA 44	161409
JIA 45	185000
JIA 46	155203
JIA 47	13498
JIA 48	87805
JIA 49	22285
JIA 50	34375
JIA 51	25984
JIA 52	46170
JIA 53	58399
JIA 54	97297
JIA 55	15189
JIA 56	121834
JIA 57	111111
JIA 58	117479