EVALUATION OF SALIVARY SOLUBLE TREM-1, IL-8, AND IL-3
AS DIAGNOSTIC MARKERS IN CHRONIC URTICARIA AT
UNIVERSITY HOSPITAL SOUTHAMPTON

by
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Abstract

Background

Chronic urticaria (CU) is a common skin disorder of the upper dermal tissue characterized by itchy, large, erythematous rashes that persist for more than 6 weeks. It can exist on its own or coexist with angioedema. A third of CU patients usually present with both hives and angioedema, whereas 30-40% present with isolated hives and 10-20% with isolated angioedema. The diagnosis of CU is difficult and complicated. Current methods are clinically based. They involve history, physical examinations, provocation/challenge, and diagnostic tests.

The association of the soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1) with CU was investigated in this study. sTREM-1 is a small soluble protein synthesised by the alternative splicing of the Trem-1 gene or the proteolytic cleavage of the TREM-1 receptor protein by metalloproteinases (MMPs). In recent years, the essential role of sTREM-1 has been noted in bacterial, viral, parasitic, and fungal infections, and has been strongly suggested that it can be used as a marker of infection.

The association of IL-8 with chronic urticaria was also investigated in this study. IL-8 is a multifunctional proinflammatory cytokine that is produced under conditions of infectious inflammatory stimulation. It is primarily released from monocytes, macrophages, fibroblasts, keratinocytes, epithelial and endothelial cells.

The association of IL-3 with chronic urticaria was also investigated in this study. IL-3 is an early activation cytokine that is expressed in hematopoietic stem cells, T cells, basophils, and many other cell types in the immune system. It is suspected to be instrumental in the exacerbation of chronic urticaria symptoms. It is associated with the upregulation of CD203c as well as CD69 expression that results in basophil activation and histamine release. IL-3 is also released as a mediator by activated mast cells undergoing degranulation.

It was hypothesized that there is significantly higher salivary sTREM-1, IL-8, and IL-3 levels in chronic urticaria patients compared to normal control subjects and that sTREM-1 levels are correlated with salivary IL-8 and are predictive of CU.
Aim
To determine and compare the levels of sTREM-1, IL-8, and IL-3 in the saliva of healthy subject controls and patients with chronic urticaria.

Method and Materials
A total of 77 subjects participated in the pilot case-control study. 43 subjects had CU (13 males and 30 females with chronic urticaria, with or without angioedema) and 34 subjects were normal controls. The levels of the salivary sTREM-1, IL-8, and IL-3 were analysed using an enzyme-linked immunosorbent assay (ELISA).

Results
The total levels of salivary sTREM-1 ($p<0.05$) and IL-8 ($p<0.0001$), but not IL-3, were significantly higher in CU patients than those of the control group. The median (25th-75th percentile) value for salivary sTREM-1 in healthy controls was 16.41 pg/ml (16.41-37.28 pg/ml) and for patients with chronic urticaria was 32.0 pg/ml (16.41-185.7 pg/ml). The median (25th-75th percentile) value for salivary IL-8 in healthy controls was 127 pg/ml (58-164 pg/ml) and for patients with chronic urticaria was 232.0 pg/ml (148.8-557 pg/ml). The median (25th-75th percentile) value for salivary IL-3 in healthy controls was 5.230 pg/ml (5.230-39.63 pg/ml) and for patients with urticaria was 5.230 pg/ml (5.230-65.10 pg/ml). The ROC curve for salivary sTREM-1 in untreated patients was highly significant ($p=0.0034$), area under the ROC curve 0.7645, std. error 0.07825, 95% confidence interval 0.6113 to 0.9181. The ROC curve for salivary IL-8 was also significant, ($p=0.0001$), area under the ROC curve 0.796, std. error 0.052, 95% confidence interval 0.69 to 0.898. The ROC curve for salivary IL-3 was not significant, ($p=0.9598$), area under the ROC curve 0.5035, std. error 0.06868, 95% confidence interval 0.3689 to 0.6381. The levels of the routine blood tests (ESR, CRP, FBC, C3C4, and Vitamin D) were normal for CU patients with Vitamin D on the lower side. There were no blood tests for the healthy controls. There were no significant correlations between salivary IL-8 and sTREM-1 or between salivary IL-8, sTREM-1, and blood biomarkers (ESR, CRP, C3, C4, Vitamin D) in the patient group.

Conclusion
The data from this study showed raised salivary sTREM-1 and IL-8 in chronic urticarial patients compared to normal controls. These elevated levels of salivary sTREM-1 and IL-8 suggest the persistent presence of oral bacterial infection in CU patients, although no
infection analysis was done in the patients in this study. sTREM-1 and IL-8 may play a key role in the host response to infection in the oral cavities of CU patients. The data also showed that there was no significant difference in salivary IL-3 of healthy subjects and chronic urticaria patients. The results cast a new light on biomarkers that may be added to a list of other biomarkers that are already used to aid the diagnosis of CU. Using salivary sTREM-1 and IL-8 together with the other biomarkers may therefore help to accelerate the diagnosis and treatment of chronic urticarial patients.
Declaration

I declare that whilst studying for the Doctorate in Biomedical Science at the University of Portsmouth, I have not been registered for any other award at another university. The work undertaken for this degree has not been submitted elsewhere for any other award. The work contained within this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text.

Witness Dzobo

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Dedication

I dedicate this thesis to my wife Ellen for your love, support, encouragement, and sacrifice. You took great care of me during a critical illness, surgery, post-surgery side effects, and when I was writing this thesis.
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Abbreviations

AAS- Angioedema activity score
ACE- Angiotensin converting enzyme
ACTH- Adrenocorticotropic hormone
AE-QoL - Angioedema quality of life
AKT- Ak strain transforming
AML- Acute myeloid leukaemia
aMMP-8 - Activated matrix metalloproteinase-8
ANOVA- Analysis of variance
Anti- FcεRI- Anti-FcepsilonRI
Anti-GPC - Anti-gastric parietal cells
Anti-IgE- Anti-immunoglobulin E
Arg- Arginine
ASST- Autologous Serum Skin Test
Anti-TPO- Anti-thyroid peroxidase
AUC- Area under the curve
BAL- Bronchoalveolar lavage
BAT- Basophil Activation Test
βc - Beta common
BHRA- Basophil histamine release assay
BSA- Bovine serum albumin
BSACI - British Society for Allergy and Clinical Immunology
C- Chemokines
CA2+ - Calcium ion
CD- Cluster of Differentiation
CD18- Cluster of differentiation 18
CD29- Cluster of differentiation 29
CD45- Cluster of differentiation 45
CD63- Cluster of differentiation 63
CD80- Cluster of differentiation 80
CD203c- Cytoplasmic Cluster of differentiation 203
CINDU- Chronic inducible urticaria
CIU- Chronic idiopathic urticaria
CLSI- Clinical laboratory standards institute
CML- Chronic myeloid leukaemia
COX-1- Cyclooxygenase-1
COX-2- Cyclooxygenase-2
CRH- Corticotropin-releasing hormone
CRP- C- Reactive Protein
CsA- Cyclosporin A
CSDH- Chronic subdural hematoma
CSU- Chronic spontaneous urticaria
CT- Computed tomography
CU- Chronic urticaria
CU-QoL - Chronic urticaria quality of life
CV- Coefficient of variation
C1q- Complement component 1q
C3- Complement component 3
C4- Complement component 4
C5A- Complement component 5A
C5aR1 – Complement factor 5a receptor 1
C5aR2- Complement factor 5a receptor 2
DAMPS- Danger-associated molecular patterns
DAP12- Dnax activating protein 12
DBMS- Doctorate of Biomedical Sciences
DCs- Dendritic cells
DNA- Deoxyribonucleic acid
DPU- Delayed pressure urticaria
DSG- Deoxyspergualin
EAACI- European Academy of Allergy and Clinical Immunology
ECI- Effective chemotactic index
EDF- European Dermatology Forum
EDTA- Ethylenediaminetetraacetic acid
ELISA- Enzyme Linked Immunosorbent Assay
E-NPP3- Ectonucleotide pyrophosphatase 3
ENT- Ear, nose, and throat
ERK - Extracellular Signal-Regulated Kinases
ESR- Erythrocyte sedimentation rate
FA-AML- Fanconi Anaemia-acute myeloid leukaemia
FBC- Full Blood Count
FceRI- Fragment crystallizable (region) epsilon Receptor /High-affinity IgE receptor
FCeRIα- FcepsilonRIalpha
FK506- Tacrolimus
GAGS- Glycosaminoglycans
GA2LEN- Global Allergy and Asthma European Network
GCF- Gingival crevicular fluid
Glu- Glutamic acid
GM-CSF- Granulocyte-macrophage colony stimulating factor
GPCR- G protein chemokine receptor
HCGF- Haematopoietic cell growth factor
Hp- Helicobacter pylori
HRA- Health research authority
HUV- Hypocomplementaemic urticarial vasculitis
H2O2- Hydrogen peroxide
IgE- Immunoglobulin E
IgG- Immunoglobulin
IL-1β- Interleukin-1beta
IL-3- Interleukin-3
IL-3Rα - Interleukin-3 receptor alpha
IL-4- Interleukin-4
IL-5- Interleukin-5
IL-6- Interleukin-6
IL-8- Interleukin-8
IL-13- Interleukin-13
IRAK- Interleukin-1 receptor-associated kinase
ITAM- Immunoreceptor tyrosine-based activation motif
kb- Kilobase
kDa- Kilodalton
K2 EDTA- Potassium Ethylenediaminetetraacetic acid
LTC4- Leukotriene C4
LTD4- Leukotriene D4
LTE4- Leukotriene E4
Leu- Leucine
LR- likelihood ratio
LPS- Lipopolysaccharides
MAMPS- Microbe-associated molecular patterns
MAPK -Mitogen-Activated Protein Kinases
MCGF- Mast cell growth factor
M-CSF- Multi-colony stimulating factor
MDA5- Melanoma differentiation-associated protein 5
MEK- Mitogen-activated protein kinase
MI- Motility index
MMP-9- Matrix metalloproteinase-9
mM- Millimolar
ml- Millilitre
mTREM-1- Membrane triggering receptor expressed on myeloid cells-1
n- Number
Na – Sodium
NaCl- Sodium Chloride.
NIK- NF-kB-inducing kinase
NFAT- Nuclear factor of activated T-cells
NF-κB- Nuclear factor kappa-light-chain-enhancer of activated B cells
NF-Kb- Nuclear factor kappa B
ng- Nanogram
nm – Nanometre
NOD1- Nucleotide-binding oligomerization domain-containing protein 1
NOD 2- Nucleotide-binding oligomerization domain-containing protein 2
NPV- Negative predictive value
NLRP1- NOD-Like Receptor Family Pyrin Domain Containing 1
NLRP3- NOD-Like Receptor Family Pyrin Domain Containing 3
NLR- NOD-like receptor
NSAIDs- Nonsteroidal anti-inflammatory drugs
OH- Hydroxide ion
OMA- Omalizumab
PAF - Platelet-activating factor.
PBS- Phosphate-buffered saline
PCT- Procalcitonin
PCR- Polymerase chain reaction
PE- Pulmonary embolism
pg- Picogram
PGS- Post graduate seminar
pH- Potential/power of hydrogen
PI- Protease inhibitor
PLC-γ - Phosphoinositide phospholipase C gamma
PI3K- Phosphoinositide 3-kinases
PKC- Protein kinase c
PPV- Positive predictive value
PRR- Pattern recognition receptor
PSF- Persisting cell-stimulating factor
QC- Quality control
QoL- Quality of life
R & D – Research and development
REC- Research ethics committee
RIG-I - Retinoic acid-inducible gene-I
ROC- Receiver operating characteristic
SA- Salicylic Acid
SD- Standard deviation
SEM- Standard error mean
sgAH- Secondary generation H1- Antihistamine
SLE- Systemic lupus erythematosus
SOP- Standard operation procedure
SPSS- Statistical Package for the Social Sciences
STAT- Signal transducer and activator of transcription
STAT3- Signal transducer and activator of transcription 3
STAT5- Signal transducer and activator of transcription 5
Std- Standard
sTREM-1- soluble triggering receptor expressed on myeloid cells-1
SYK- Spleen tyrosine kinase
TCR- T cell receptor
TH2- T helper cell 2
TLR2 - Toll-like receptor 2
TLR3 - Toll-like receptor 3
TLR4 - Toll-like receptor 4
TLR5 - Toll-like receptor 5
TLR7 - Toll-like receptor 7
TLR8 -Toll-like receptor 8
TLR9 - Toll-like receptor 9
TLT-1-TREM-like transcript -1
TLT-2- TREM-like transcript-2
TMB- Tetramethylbenzidine
TNF-  Tumor necrosis factor
TNF-α-  Tumor necrosis factor-alpha
TREM-1-  Triggering receptor expressed on myeloid cells-1
TREM-2 -  Triggering receptor expressed on myeloid cells-2
TREM-3-  Triggering receptor expressed on myeloid cells-3
UAS-  Urticaria Activity Score
UAS7-  Urticaria Activity Score-7
UCT-  Urticaria control test
UHS-  University Hospitals Southampton
UK-  United Kingdom
VEGF-  Vascular endothelial growth factor
VDBP-  Vitamin D binding protein
VIT D-  Vitamin D
VTE-  Venous thromboembolism
WAO-  World Allergy Organisation
ZAP70-  Zeta-chain-associated protein kinase 70
µg-  Microgram
µl-  Microlitre
µM-  Micromolar
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Chapter 1: Introduction

1.1 Urticaria

Urticaria is a skin condition characterized by the development of wheals (hives), angioedema, or both (Antia et al., 2018; Paudel et al., 2020; Vurgun et al., 2020; Zuberbier et al., 2018), and is a common clinical condition seen in dermatology departments. It affects between 8% and 20% of the world population (Darlenksi et al., 2014; Zuberbier et al., 2018; Zuberbier et al., 2014; Zuberbier et al., 2010). Mast cells and basophils play a pivotal role in the pathophysiology of urticaria, with the mast cell being the primary effector cell, and the main mediator is histamine (Figure 1)(Pels, 2015).

Pathophysiology of Mast Cell Degranulation

Figure 1: The pathophysiology of mast cell degranulation.

The mast cell is activated by an allergen (food, venom, or latex) cross-linking two IgE molecules bound to high affinity Fce receptors. It is also activated by stress, nutrition, drugs, infection, and inflammation. In addition to histamine, activated mast cells release lipid mediators such as platelet-activating factor (PAF), leukotrienes, prostaglandins, and peptide cytokines (Zuberbier et al., 2018), which cause sensory nerve activation, vasodilation, plasma extravasation as well as neutrophils, and/or eosinophils, macrophages...
and T cells recruitment to urticarial lesions (Haas et al., 1998; Ito et al., 2011; Kay et al., 2015; Kay et al., 2014a; Zuberbier et al., 2018).

This mixed inflammatory perivascular infiltrate is highly variable and does not lead to vessel wall damage as observed in urticaria vasculitis (Peteiro & Toribio, 1989; Zuberbier et al., 2018). It results in a rapid onset of localized wheals (Figure 2), angioedema (Figure 3), or both (Darlenksi et al., 2014; Jurakić Tonći et al., 2009).

![Figure 2: Cutaneous urticarial wheals. Photo courtesy of UHS allergy clinicians. UHS, University Hospital Southampton.](image1)

A wheal or hive is a suddenly formed elevation of the skin surface (Figure 2), has a central oedema of variable size, is surrounded by reflex erythema, results in itching or sometimes a burning sensation, and has a transient nature (Petkova & Staevska, 2020; Zuberbier et al., 2018). The affected skin returns to normal usually within 30 minutes to 24 hours.

![Figure 3: Angioedema in a patient with urticaria. Photo courtesy of UHS allergy clinicians.](image2)
Angioedema (Figure 3) is the sudden marked erythematous or pale swelling of the underlying dermis and subcutaneous tissue or mucous membranes that can be painful rather than itchy (Petkova & Staevska, 2020; Zuberbier et al., 2018). Clinically, it presents as swellings on the face, oropharynx, and peripheries including genitalia (Powell et al., 2015). Angioedema takes about 72 hours to resolve (Petkova & Staevska, 2020; Yan et al., 2015). In addition to skin mast cells, basophils also contain and release histamine (Vurgun et al., 2020). Basophils contribute approximately 2.9%, whereas skin mast cells contribute approximately 11.4% of the total histamine release in urticaria patients (Zuberbier et al., 1996).

Many factors are suspected to trigger the onset of urticaria (Figure 1). These include physical stimuli, bacterial and viral infections, foods, and autoimmune disorders (Curto-Barredo et al., 2014). Clinical manifestations of urticaria are ill-defined in many cases, are most likely to be heterogeneous and diverse. It is therefore not surprising that two or more different triggers can be described as coexisting in any given patient (Zuberbier et al., 2018).

Urticaria cases are classified as either acute or chronic. Urticaria is defined as acute if the whealing persists for less than six weeks (Ben Mahmoud et al., 2011; Darlenski et al., 2014; Petkova & Staevska, 2020; Vurgun et al., 2020; Zuberbier et al., 2018; Zuberbier et al., 2014). An external cause is identified in only 50% of all acute urticaria cases. Viral infections are the main trigger, specific food and drugs being less common triggers (Petkova & Staevska, 2020). Acute urticaria is increased in atopic individuals. It is a self-limiting condition. The international consensus guidelines do not recommend extensive diagnostic procedures on acute urticaria (Zuberbier et al., 2018). Urticaria is defined as chronic urticaria if daily or almost daily wheals or angioedema are present for more than 6 weeks (Antia et al., 2018; Petkova & Staevska, 2020; Vurgun et al., 2020; Yan et al., 2015; Zuberbier et al., 2018; Zuberbier et al., 2014). Chronic urticaria (CU) is not increased in atopy. The focus of this study is on chronic urticaria because the University Hospital Southampton (UHS) allergy clinic receives many more chronic urticaria patients than acute urticaria patients. General Practitioner surgeries deal with most of the acute urticaria patients and refer CU patients to the allergy clinic because they are more complex and difficult to manage.
1.2 Chronic Urticaria

1.2.1 Definition of Chronic Urticaria
Chronic urticaria is a common skin disorder of the upper dermal tissue characterized by itchy, large, erythematous rashes that persist for more than 6 weeks (Paudel et al., 2020; Petkova & Staevska, 2020; Vurgun et al., 2020; Zuberbier et al., 2018). It can exist on its own or coexist with angioedema. A third of CU patients usually present with both hives and angioedema, whereas 30-40% present with isolated hives and 10-20% with isolated angioedema (Antia et al., 2018). The Global Allergy and Asthma European Network/European Academy of Allergy and Clinical Immunology/World Allergy Organisation/European Dermatology Forum (GA²LEN/EAACI/WAO/EDF) international guidelines recommend classifying CU per clinical manifestation as opposed to pathophysiology which was based on old guidelines (Zuberbier et al., 2018; Zuberbier et al., 2014). These guidelines subdivide CU into two subtypes for clinical use: chronic spontaneous urticaria (CSU), which is the spontaneous appearance of wheals, angioedema, or both, lasting longer than 6 weeks due to known or unknown causes and chronic inducible urticaria (CINDU), which includes demographic urticaria, cold urticaria, delayed pressure urticaria, solar urticaria, heat urticaria, vibratory angioedema, cholinergic urticaria, contact urticaria and aquagenic urticaria (Zuberbier et al., 2018; Zuberbier et al., 2014).

1.2.2 Epidemiology of Chronic Urticaria
Chronic urticaria has a prevalence of between 0.1% and 3% in the general world population (Curto-Barredo et al., 2014; Fricke et al., 2020; Paudel et al., 2020). On the global level, CU appears to be more prevalent in Asia and Latin America than in Europe and Northern America. The prevalence of CU in Asia is 1.4% (Chu et al., 2017), in Latin America is 1.5% (Balp et al., 2017), in Northern America is 0.1% (Vietri et al., 2015; Zazzali et al., 2012), and in Europe, the prevalence ranges from 0.38% to 0.8% (Balp et al., 2015; Lapi et al., 2016; Zuberbier et al., 2010). CU is more common in adults, especially between the ages of 25 and 55 years (Gaig et al., 2004). Women are affected more than men with a prevalence ratio of 4:1 (Kim et al., 2018; Lakshmikanth & Sukumar, 2016; Lapi et al., 2016; Liu et al., 2012; Mlyněk et al., 2009; Mlyněk, Maurer, et al., 2008). The high prevalence in women has often been linked to sex hormone levels. CU appears to
be less common in children than in adults (Dilek et al., 2016), with the prevalence of CU in children reported to be 0.1 to 0.3% in the UK (Ferrante et al., 2015).

1.2.3 Clinical Presentation of Chronic Urticaria

![Image](image.png)

Figure 4: Wheals on the arms and back of a CU patient
Photo courtesy of UHS allergy clinicians.

The clinical presentation involves red, swelling, and itchy plaques that can affect the arms, legs, chest, and back (Figure 4). These lesions usually resolve within 2-3 hrs without a trace. These patients sometimes present with angioedema that can also involve the respiratory tract. If respiratory tracts are involved, they may be life threatening and should be treated urgently (Kayiran & Akdeniz, 2019). In cases of angioedema, mucus membranes, such as the eyes, lips, sometimes tongue, swell with some pain and burning sensation (Figure 3).

Due to extreme pruritus, unsightly skin lesions, and symptom recurrence many patients with chronic urticaria present with a significant reduction in quality of life. These patients experience severe itching of the skin, sleep disturbances, fatigue, social isolation, emotional disorders, difficult relations to work, domestic activities, home relationships, sexual life, hobbies, and holidays (Ben Mahmoud et al., 2011; Darlenski et al., 2014). The impact of CU on quality of life is comparable to that suffered by patients with severe
coronary artery disease awaiting bypass (ODonnell et al., 1997; Zuberbier et al., 2018). Besides, the disease has a considerable impact on direct and indirect health-related costs to the patients and the healthcare system (Antia et al., 2018). It is well established that patients with chronic urticaria are frequent users of medical services, such as primary care clinics or hospital emergency departments, and outpatient allergy clinics (Confino-Cohen et al., 2012).

In most cases, chronic urticaria is self-limiting, typically lasting for months to several years. On average, the duration of the disease is between three to five years. The remission of the disease is achieved in approximately 25% after 3 months, 50% after a year, 80% after 3 years, and 90% after 5 years (Eun et al., 2019). In exceptional cases, 20% of these patients, symptoms may be noticed for more than 10 years (Maurer, Weller, et al., 2011). Sadly, a relapse occurs in half of the patients at least once after remission (Beltrani, 2002).

1.2.4 Pathology of Chronic Urticaria.

Several etiologic factors have been associated with CU over time, including thyroid diseases, pseudo-allergens, actual allergens, Helicobacter pylori infection, other infections/infestations, and autoimmunity/autoreactivity (Criado et al., 2015). Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are also known to exacerbate the pre-existing CU (Kowalski et al., 2013). Circulating or tissue-resident mast cells and/or tissue recruited basophils play an important role in the pathophysiology of chronic urticaria by releasing histamine (Brodell, 2008; Engin et al., 2020), Figure 1. Histamine is the main inflammatory mediator most clearly associated with chronic urticaria. The cutaneous mast cell is regarded as the main effector cell in the production of wheals seen in CU patients (Curto-Barredo et al 2014). Previously, basophils were only regarded as bystanders and served as biomarkers in some chronic urticaria subsets. In recent years, the role of basophils has been investigated in CU. Basophils have now been found to play an active role (Figure 5) and to be present in the leukocyte infiltrate in skin biopsies of CU lesions (Ying et al., 2002). It is now known that basophils contain and release histamine contributing about 2.9% of the histamine seen in urticaria (Vurgun et al., 2020; Zuberbier et al., 1996). Reduced circulating basophil numbers have also been correlated with heightened CU disease activity (Grattan et al., 2003; Grattan et al., 1997).

Nevertheless, much effort has focused on understanding the mechanisms responsible for the mast cell- histamine release axis in chronic urticaria (Figures 5 and 6). Briefly, when
the mast cell is activated it undergoes degranulation with the release of histamine and other mediators such as platelet-activating factor (PAF), tumour necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (IL-3, IL-4, IL-5, IL-6, IL-8, IL-13) and leukotrienes (Leukotriene C4 (LTC4), Leukotriene D4 (LTD4), Leukotriene E4 (LTE4)) (Curto-Barredo et al., 2014; Zuberbier et al., 2014). The release of histamine and these other mediators result in sensory nerve activation, vasodilation, and plasma extravasation as well as inflammatory cell recruitment to urticarial lesions (Haas et al., 1998; Kay et al., 2015; Kay et al., 2014b).

Initiations of chronic urticarial lesions or flares are likely due to more than one trigger for most patients. Most chronic urticarial cases (as opposed to acute urticaria) have no known allergen or trigger (Sun et al., 2011).

Figure 5: Pathogenesis of chronic inducible and spontaneous urticaria. The skin mast cells or basophils degranulate and release histamine and other proinflammatory mediators. The degranulation of these cells in some CSU patients is caused by autoantibodies directed against a subunit of the high-affinity IgE receptor, FcεRI, or to IgE itself. Other potential activators of mast cells or basophils that are relevant to CSU involve autoantigens and IgE directed against these autoantigens, as well as opioids, complement factors (C5a), cytokines, neuropeptides, drugs, stress, pressure, and temperature. Adapted from Chang et al (2015)
8

Figure 6: Mast cell activation in affected skin.
In a CSU patient, the inflammatory manifestation of mast cells in the affected skin is the final common pathway. Identifiable external triggers such as cold, heat, sunlight, and pressure are the triggers that activate the mast cell to degranulate in CINDU. For CSU, the primary causative factors (anti-FCER1, anti-IgE, autoreactive IgE, anti-TPO) that activate the mast cell to degranulate arise internally. There are remaining cases of CSU whose internal abnormalities have not been identified. Adapted from Chang et al., 2015.

1.2.5 Chronic Urticaria and Autoimmune Disease
Chronic spontaneous urticaria (CSU) accounts for 70% of all cases (Curto-Barreto et al., 2014). 45% of CSU is autoimmune whereas the rest is of unknown cause. Patients with the unknown trigger are classified as having chronic idiopathic urticaria (CIU) and treatment is focused on symptom control. Hashimoto's thyroiditis and Graves' disease are known to be associated with idiopathic CU (Leznoff et al., 1983; Leznoff & Sussman, 1989). Hashimoto thyroiditis is an autoimmune thyroid disease characterized by increased thyroid volume, lymphocyte infiltration of parenchyma, and the presence of antibodies specific to thyroid antigens (Ralli et al., 2020). Graves' disease is a systemic autoimmune disease directly caused by circulating autoantibodies that bind to the thyrotropin receptor, subsequently inducing the production and release of thyroid hormone, the proliferation of thyrocytes, and enlargement of the thyroid gland (Kahaly, 2020). Studies have reported that antithyroid antibodies are detected in 27% of patients with idiopathic CU compared to 6% of the general population and 19% of these patients have abnormal thyroid function (Zauli et al., 2002). Also, there is a close association between chronic urticaria and autoimmune disease in CU patients. Autoimmune disease accounts for 30% to 50% of all cases of chronic urticaria hence the name chronic autoimmune urticaria (Curto-Barreto et al., 2014; Luo et al., 2018). The patients with chronic autoimmune urticaria have functional autoantibodies to the alfa subunit of the FcεRIα (FcεR1α) and antibodies to the
IgE of the mast cells (see Figures 5 and 6) (Chang et al., 2015; Curto-Barredo et al., 2014). It is interesting to note that in this condition the cutaneous mast cells are permanently activated due to the presence of functional IgG antibodies to the alfa subunit of the FCεR1 or to the IgE of the mast cells. Basophils also express FCεR1 (Figure 5) and a fall in basophil levels is usually detected in patients with chronic autoimmune urticaria (Grattan et al., 2003).

1.2.6 Chronic Urticaria and Drugs

CU can be exacerbated by other factors not related to a trigger. Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are known to exacerbate the pre-existing chronic spontaneous urticaria (Kowalski et al., 2013; Sánchez-Borges et al., 2015) and the incidence of intolerance in the general population is 0.3% (Grattan, 2003). NSAID-induced urticaria/angioedema have been reported to make up 50% of overall NSAIDs hypersensitivity (Kowalski et al., 2013). Also, aspirin-induced CSU exacerbations have been reported in 20-40% of all patients (Grattan, 2003). Aspirin and NSAIDs inhibit cyclooxygenase (COX)-1 and inducible COX-2, thus diverting arachidonic acid metabolism towards the 5-lipoxygenase metabolic pathway in eosinophils (Borzova & Grattan, 2019; Nagelschmitz et al., 2014; Skypala et al., 2015). This modulation is associated with the overproduction of cysteinyll-leukotrienes LTC4, LTD4, and LTE4. These cysteinyl leukotrienes promote mast cell or eosinophils degranulation and chemotaxis which results in vasodilation and oedema seen in CU. This process is not IgE mediated.

1.2.7 Chronic Urticaria and Salicylates

Natural salicylates/salicylic acid (SA) is related to aspirin, but they lack the acetyl group. Salicylates inhibit COX-1 activity leading to reduced prostaglandin synthesis.

A high salicylate diet is suspected to worsen symptoms of CU in the same way as taking aspirin (Gumunyu et al., 2017; Wright & Minford, 1999). It is proposed that salicylates increase IL-3 synthesis? (Sánchez-Borges et al., 2015) which lowers the mast cell and basophil threshold for degranulation.

1.2.8 Chronic Urticaria and Diet

Nutritional deficiency of micronutrients such as iron, Vitamin B12, and Vitamin D has been suggested to play an important role in the etiology of CU. Previous studies have
reported hyposideremia (iron deficiency) in CU patients poorly responsive to usual treatments and oral iron therapy was reported to correct the deficiency (Guarneri et al., 2014; Sharma, 2010; C. H. Wu et al., 2015). UHS allergy clinic clinicians have confirmed, anecdotally, that it is common to see low ferritin with normal FBC in patients with chronic urticaria. Also, previous studies have reported low vitamin B12 in CU patients who responded to the correction of the deficiency (Kasperska-Zając et al., 2017; Mete et al., 2004; C. H. Wu et al., 2015).

High levels of anti-gastric parietal cells (anti-GPC) antibodies and anti-thyroid autoantibodies were also detected in CU patients (Mete et al., 2004) suggesting that the low vitamin B12 in these CU patients may be autoimmune in nature. UHS allergy clinicians have also confirmed, anecdotally, that it is also common to find low vitamin B12 with normal FBC in some CU patients.

In addition to iron and vitamin B12 deficiency, some studies have reported a significant number of patients with CU who had vitamin D deficiency and responded to correction of the deficiency (Movahedi et al., 2015; Tuchinda et al., 2018; Yuan et al., 2019). UHS allergy clinicians have also confirmed, anecdotally, that they also see a significant number of patients with CU who are vitamin D deficient.

1.2.9 Chronic Urticaria and Stress

Stress, depression, and anxiety are important factors thought to be involved in the development and aggravation of chronic urticaria (Choi et al., 2020; Mlynek, Maurer, et al., 2008; Vurgun et al., 2020). The mechanisms by which these conditions impact CU have not been fully elucidated. However, two mechanisms have been suggested (Pasaoglu et al., 2006; Yang et al., 2005). The first potential theory suggests that mast cell activation is elicited through the hypothalamic-pituitary-adrenocortical axis, the sympathetic and adrenomedullary mediators, and local skin nerves. The alternate theory suggests that stress can induce the release of cortisol and the expression of inflammatory mediators, leading to the activation of cutaneous mast cells and the development of CU (Choi et al., 2020). In addition, basophils are suspected to be activated by the stress hormones adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) in CU patients (Dyke et al., 2008). Treatment of these patients involves psychological interventions which have been tried and reported to be effective (Buffet, 2003; Schut et al.,
Patients usually report improvement in symptoms when these psychological factors are removed.

1.2.10 Physical and Chemical Stimuli
Chronic inducible urticaria (CINDU) is a group of urticarias (Table 1 and Figure 6), with a duration of 6 weeks or longer. They are also characterised by recurrent wheals, angioedema, or both, which only occur after exposure to definite external triggers (Magerl et al., 2016; Zuberbier et al., 2018). The triggers that cause signs and symptoms in these patients are mainly physical or chemical stimuli.

The physical stimuli include pressure (in delayed pressure urticaria), radiation (in solar urticaria), friction (in symptomatic dermographism), temperature (in cold and heat urticaria), and vibration (in vibratory angioedema) (Maurer et al., 2019; Yan et al., 2015). Delayed pressure urticaria (DPU) is characterised by deep swellings that are seen at sites of pressure application on the skin. Mast cells are involved through non-immunologic mechanisms and are not IgE mediated. With temperature, the cold induced cholinergic urticaria is the most prevalent. It is caused by exercising in the cold environments. It is characterised by generalised urticaria and localised cold urticaria, in which only certain areas of the body urticate with cold contact (Maurer et al., 2018; Maurer, Magerl, et al., 2013) The chemical triggers include water (in aquagenic urticaria), sweat (in cholinergic urticaria) and urticariogenic chemical compounds (in contact urticaria) (Maurer et al., 2019).

1.2.11 Chronic Urticaria and Bacterial Infections.
The connection between urticaria and infections has been discussed for many years (Minciullo et al., 2014a). Many bacterial, parasitic, viral, and fungal infections have been implicated to be the underlying causes of urticaria. These include Helicobacter pylori, Streptococcus, Staphylococcus, Yersinia enterocolitica, Mycoplasma pneumonia, Salmonella, Brucella, Chlamydia pneumonia, Versinia, Giardia Lamblia, Hepatitis Virus, Norovirus, Parvovirus B19, Entamoeba spp and Blastocytis spp (Imbalzano et al., 2016; Kolkhir et al., 2016; Minciullo et al., 2014a, 2014b; Wedi et al., 2004; Zuberbier et al., 2018). However, a causal relationship with underlying or precipitating infection is still difficult to establish in CU patients.
More research is still needed to make definitive conclusions regarding the role of infection in chronic urticaria. In many cases where aetiology is suspected, infections are the underlying persistent cause, aggravating factor, or bystander most associated with CU (Wedi et al., 2004). Mast cells and basophils have receptors of complement component 5a (C5a). When the complement is activated by infection C5a augments histamine release from basophils through the stimulation of anti-FCεR1 autoantibodies (Yanase et al., 2021).

The incidence and impact of these infections vary between different patient groups and regions. For example, viral hepatitis is a common cause of CU in southern European countries but is rarely associated with the condition in northern Europe (Petkova & Staevska, 2020). Anisakis Simplex, a sea fish nematode, is suspected to be a cause in regions where raw fish are consumed (Foti et al., 2002).

The relevance of Helicobacter Pylori, dental, ear, nose, and throat infections also appear to vary between patient groups (Curth et al., 2015; Dionigi et al., 2016; Minciullo et al., 2014a; Rasooly et al., 2015; Shabrawy & Gharib, 2016).

1.2.11.1 Helicobacter Pylori Infection

Helicobacter pylori infection is thought to be the most common chronic bacterial infection that affects humans (Peek & Blaser, 2002), with the prevalence rate in the general population estimated to be around 50% in developing countries (Chiu et al., 2013; Suerbaum & Michetti, 2002). There is substantial evidence that suggests Helicobacter pylori infection is associated with CU (Castillo Reguera et al., 2012; Fukuda et al., 2004; Gu et al., 2015; Magen et al., 2007; Minciullo et al., 2014b; Wedi et al., 2009), even though there are conflicting reports in the literature regarding this association (Curth et al., 2015; Moreira et al., 2003; Shakouri et al., 2010). A recent study showed clear evidence of the association of CU with Helicobacter pylori infection (Tan et al., 2016). The study reported that a 21-35 kDa mixed protein component from Helicobacter pylori induces mast cell degranulation. As a result of this finding, the eradication of Helicobacter pylori is recommended as part of routine chronic urticaria management. This is supported and proven by research that showed Helicobacter pylori eradication demonstrated statistically significant benefits compared to untreated patients or Helicobacter negative controls without urticaria (Chiu et al., 2013; Wedi et al., 2009). However, an early study conflicts with these recommendations (Goga et al., 1988). Even though this association is debatable, clinicians are highly recommended to consider bacterial agents in the diagnostic
examination of patients with chronic urticaria as the eradication of the infection could lead to the resolution of chronic urticaria (Zuberbier et al., 2018).

1.2.11.2 Periodontitis

Periodontitis is an irreversible common chronic oral inflammatory disease affecting the tooth-supporting tissues including the gingiva, periodontal ligament, and alveolar bone (Willi et al., 2014). It is caused by multifactorial infectious diseases that manifest clinically by the destruction of the supporting tissues of the teeth (Bostanci et al., 2007). Periodontitis is regarded as the most prevalent chronic inflammatory disease in humans affecting around 20-50% of the population worldwide (Räisänen et al., 2020; Sukriti et al., 2020). Approximately 5-20% of patients with severe periodontitis end up losing their teeth globally (Räisänen et al., 2020). Children and adolescents are not spared as they may also suffer from the periodontal loss.

More than 700 bacterial species are suspected to colonise the oral cavity (Aas et al., 2005), however, only a small number cause periodontal diseases (Socransky et al., 1998). Early studies indicate that a group of bacteria rather than a single species is associated with periodontal tissue destruction (Belibasakis et al., 2014; Bodet et al., 2006; Willi et al., 2014), with *Porphyromonas gingivalis* being reported as the main component of the pathogenic microbiota of periodontal diseases (Holt et al., 1999; Slots & Ting, 1999; Socransky et al., 1998). Campylobacter rectus is also implicated in various forms of periodontal diseases (Tanabe et al., 2003).

It is noteworthy to understand the pathological process of periodontitis. Periodontitis is mainly attributed to localised inflammation resulting from interactions between the host and the pathogenic microbiota (Belibasakis et al., 2014; Willi et al., 2014). Interestingly, this study identifies the association between dental infection (periodontitis) and chronic urticaria. Ear, nose, and throat as well as dental infections are suspected to associate with CU and the incidence tends to vary between patient groups (Tarbox et al., 2011). Studies have provided robust evidence that infection involving teeth and sinuses is frequently found in chronic urticaria patients (Ertam et al., 2007; Tarbox et al., 2011). It has been shown that treatment with antibiotics results in the resolution of urticaria (Ertam et al., 2007). Worryingly, very few clinicians take time to examine the mouth and teeth of patients with chronic urticaria. Oral and gum Infection may not be obvious as shown in
Figure 7 shows teeth and gums of some CU patients who visited the UHS allergy clinic. Often these patients attend allergy clinic and other primary healthcare facilities for routine allergy appointments and without presenting clearly identifiable signs or symptoms of sinus and/or gum infections resulting in this tractable trigger for urticaria being missed.

Figure 7: Teeth of some of the patients visiting the UHS allergy clinic. Pictures are courtesy of the UHS allergy clinicians.
1.3 Chronic Urticaria: Management/Treatment
Chronic urticaria is a very difficult target disease to manage and treat despite many modern medical and technological advances at our disposal. It is a formidable challenge that usually leaves both clinicians and patients frustrated hence the need for good clinical practice which includes individualised care of patients. The EAACI/GA (2) LEN/EDF/WAO guidelines (Zuberbier et al., 2018; Zuberbier et al., 2014) reached a consensus that defines the treatment objective of chronic urticaria patients, which is to successfully bring about complete resolution of signs (hives and angioedema) and relief of symptoms (pruritus and pain) as quickly as possible (Giménez-Arnau et al., 2016). Hence, a tailored approach that takes into consideration the duration of the disease, a clinical course in previous flares, the characteristics of wheals and their locations, the presence or not of angioedema, the severity of symptoms, interference with quality of life, the co-occurrence of several types of urticaria, comorbidity and response to previous treatments is recommended in the guidelines (Zuberbier et al., 2018; Zuberbier et al., 2014).

1.3.1 Chronic Urticaria: Routine Diagnostic Tests
The diagnosis of CU is difficult and complicated. Current methods are clinically based. They involve history, physical examinations, provocation/challenge, and diagnostic tests. Severity assessments using UAS7, AAS, CU-Q2Ol, AE-QoL, and UCT are also applied (Petkova & Staevska, 2020). The most used and recommended routine diagnostic tests are erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), full blood count (FBC) (Criado et al., 2015; Zuberbier et al., 2018).

1.3.1.1 C-Reactive Protein
CRP is an acute-phase protein mainly synthesised by hepatocytes in the liver and is regulated by cytokines, interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α) (Bennett & Plum, 1996; Corey-Bloom et al., 2020; Del Giudice & Gangstad, 2018; Ebersole & Cappelli, 2000).

CRP is routinely used as a marker of bacterial infections. It is an extremely sensitive, but a non-specific acute phase protein (Aguiar et al., 2013; Pay & Shaw, 2019). CRP levels in plasma are normally low (below 10 mg/l) but it is known that bacterial infections cause a rapid elevation of CRP levels within hours of infection. It outperforms ESR in terms of responsiveness and specificity for inflammation.
1.3.1.2 Erythrocyte Sedimentation Rate

ESR is a test used to investigate pathological conditions. It is frequently used in laboratories with normal ESR indicating the absence of serious pathology.

Elevated ESR is often suspected of indicating an infection or a malignant disease. It is a non-specific method, and its levels are upregulated in many non-infectious conditions such as stroke and coronary artery disease. In general, CRP and ESR tests are rarely outside the normal range and very rarely influence the diagnosis and management of CU (Tarbox et al., 2011).

The selection of a biomarker for the identification of bacterial infections, in general, has always been a challenge. This has resulted in widespread unnecessary ordering of laboratory tests in the healthcare system leading to increased expenditure without the demonstrable benefit (Detsky & Verma, 2012). When patients with fever or other inflammatory conditions are evaluated, a CRP and an ESR are commonly ordered in tandem, and yet there is no evidence-based justification for this practice (Kainth & Gigiotti, 2014), although some reports indicate that the combination of these two biomarkers is potentially useful in diseases such as systemic lupus erythematosus (SLE) (dos Santos et al., 2013; Keenan et al., 2008; Littlejohn et al., 2018). SLE is an autoimmune disease in which the immune system attacks its own tissues causing widespread inflammation and tissue damage in the affected organs (Zhou, Xu, et al., 2020).

For a long time, the use of these two simple tests, CRP and ESR has been employed to single out chronic urticaria patients with success to a certain degree hence the compelling need for a definitive test for these patients.

1.3.1.3 D-Dimer

More recently, several biomarkers related to CU prognosis and therapeutic response have been described (Deza et al., 2018). Elevated D-dimer levels have been found in CU patients with insufficient clinical response to antihistamine treatment (Asero, 2013; Takahagi et al., 2010). D-dimer is a final plasmin-mediated fibrin degradation product consisting of two covalently bound fibrin D domains, cross-linked by factor XIII during clot formation (Darlen ski et al., 2014; Favresse et al., 2018; Linkins & Takach Lapner, 2017; Riley et al., 2016). Measurement of D-dimers is normally used to rule out venous thromboembolism (VTE) and pulmonary embolism (PE), as well as an aid in the diagnosis.
of disseminated intravascular coagulopathy. However, high D-dimer values are associated with several other conditions including inflammation (Lapić et al., 2020).

1.3.1.4 Fibrinogen
Also, increased levels of fibrinogen were considered to be a predictor of poor response to antihistamines in CU patients (Kolkhir et al., 2017). Fibrinogen, synthesized by hepatocytes in the liver and circulating in the bloodstream, is a pleiotropic protein with essential roles in cell-matrix interaction, haemostatic and inflammatory systems (Zhou, Liang, et al., 2020).

1.3.1.5 Complement Components
More interesting is a finding that suggested increased complement C5a levels indicate higher CU disease activity and longer duration of wheals (Huilan et al., 2015). Complement factor C5a is an integral constituent of the complement cascade critically involved in the innate immune response, and it exerts its functions via two distinct receptors, C5aR1 and C5aR2 (Pandey et al., 2020). C5a is a potent inflammatory peptide, commonly referred to as an anaphylatoxin, as it possesses proinflammatory, spasmogenic, and chemotactic properties and its sustained plasma levels can lead to excessive inflammation as observed in sepsis (Manthey et al., 2009; Ward, 2004). Also, complement factors C4 and C1q are helpful to distinguish CU from urticarial vasculitis. Urticarial vasculitis is a rare clinico-pathologic entity characterised by urticarial lesions (Figure 8) that persist for more than 24 hrs and have histologic features of leukocytoclastic vasculitis (Peroni et al., 2010; Pinto-Almeida et al., 2013). The cutaneous lesions have burning or painful sensations and pruritus that resolve with residual hyperpigmentation (Chen & Carlson, 2008; Russell & Gibson, 2006). There are two types of patients observed in this group: those with normal complement and those who have hypocomplementaemic urticarial vasculitis (HUV). C4 and C1q levels have been observed to be decreased in HUV (Pinto-Almeida et al., 2013). In addition, some studies have reported elevated C1q antibodies in HUV regarding it as an autoimmune disease (AlHermi et al., 2017; Jayakanthan et al., 2017).
1.3.1.6 Autologous Serum Skin Test

Non-routine antibody-based tests are also selected in certain CU patients, for example, the autologous serum skin test (ASST) in patients with CSU (Criado et al., 2015). ASST is a simple in vivo non-specific test that tests the presence of autoantibodies against the IgE receptor. It is used in clinical setups for the detection of mast cells and basophil histamine-releasing activity (Al-Hamamy et al., 2013; Baumann et al., 2021) and about 30% to 50% of CU patients show a positive response against intradermal injection of their own serum (Confino-Cohen et al., 2012). It also assesses the presence of serum histamine-releasing factors of all types, not just antibodies. It has a sensitivity of approximately 70% and a specificity of 80% (Al-Hamamy et al., 2013). ASST is helpful to detect autoreactivity but is not diagnostic for CU. The downside of this test is the potential risk of accidental infection if the patient is injected with unknown serum by mistake, and for this reason not used routinely in the clinic.

1.3.1.7 The Basophil Histamine Release Assay and the Basophil Activation Test

In some specialised centres, more specific laboratory tests are available and are used for autoantibody testing. These include the in-vitro laboratory testing using the basophil histamine release assay (BHRA) and the basophil activation test (BAT) by flow cytometry. BHRA and BAT tests have different methodologies but they both measure the basophil responses to an activating signal. In the BHRA, serum from the patient is incubated with basophils from a healthy donor. Any resulting histamine released is expressed as a
percentage of total histamine. The patient serum contains the histamine-releasing factors, IgG antibodies (IgG anti-FcεRI and IgG anti-E autoantibodies) specific for the high-affinity IgE receptor (FcεRI) present on mast cells and basophils (Greaves, 1995; Hide et al., 1993; Tong et al., 1997) or for IgE (Grattan et al., 1991; Gruber et al., 1988).

In the BAT test, flow cytometry is used to detect CD203c and CD63 expressed on activated basophils (Boumiza et al., 2005). The test assesses the level of basophil activation which is mediated primarily by cross-linking of the high-affinity IgE receptor FcεRIα. CD203c (ectonucleotide pyrophosphatase: E-NPP3) is a surface marker observed uniquely on basophils and mast cells that is upregulated by anti-IgE antibodies and allergens (Vasagar et al., 2006; Ye et al., 2014). CD63 is a member of the transmembrane-4 superfamily and is rapidly mobilized on the basophil surface by IL-3, anti-IgE, and allergen (Vasagar et al., 2006). CD63 is anchored in the basophilic granule membrane (which contains histamine) and its exposure to the outside of the cells reflects cell degranulation due to fusion between granules and plasma membranes (Boumiza et al., 2005).

Despite the BHRA and BAT being recognized as the gold standard for some forms of CSU the assays are not fully standardised and are time-consuming, sometimes only being used for research purposes (McGowan & Saini, 2013). CRP and ESR are useful, particularly in excluding significant infection, inflammatory urticarial and associated autoimmune conditions, although rare. Often using these usual markers of infection is not helpful in most CU patients, particularly those with dental and sinus bacterial infections as the levels would appear normal because the infection is localised/focal.

### 1.3.1.8 Saliva Tests in Chronic Urticaria

Biomedical scientists and clinicians motivated by the need for a better diagnostic assay in CU patients have been investigating different diagnostic assays for quite some time now. The enzyme linked immunosorbent assay (ELISA) technique was used in this current study to measure potential salivary biomarkers of CU. This use of the ELISA technique in CU has enormous potential patient and financial benefits. The ELISA technique is easy, rapid, inexpensive, and can be performed for small batches of specimens. The blood sample has been the most used sample to identify diseases and monitor the progress of treatment. However, there is a growing interest in the use of saliva for diagnosis, treatment, and monitoring of diseases in different fields (Giannobile et al., 2011).
Saliva is a clear, slightly acid mucinous-serous secretion (Kubala et al., 2018). It contains mainly water-95%, proteins-0.3%, and inorganic and organic substances-0.2% (Liu & Duan, 2012). The inorganic components in saliva are mostly electrolytes, whereas enzymes, hormones, and proteins are the organic components present (Chiappin et al., 2007; Humphrey & Williamson, 2001). Ninety percent of human saliva is produced by the three large salivary glands- the parotid, submandibular and sublingual, and the remaining 10% is produced by about 200-400 small fine salivary glands (Kaufman & Lamster, 2002). Healthy individuals produce approximately 0.5 to 2 litres of saliva per day (Mese & Matsuo, 2007). Usually, the rate of secretion is decreased during sleeping but increases markedly during chewing and eating (Kubala et al., 2018). Saliva has a wide variety of biological functions that are critical for the maintenance of oral health which are: (i) washes the mucus membranes of mouth, throat, and larynx, (ii) lubricates, buffers, offers antimicrobial protection, and maintains mucosal integrity (Huang, 2004), (iii) it moisturises oral tissue allowing digestion and swallowing, (iv) it moistens the mucus membranes and the teeth, (v) it protects the surface of the teeth and mucus membranes against biological, mechanical and chemical factors (Hicks et al., 2003). The blood sample is the most used sample to identify diseases and monitor the progress of treatment. However, there is a growing interest in the use of saliva in many fields of sciences such as medicine, dentistry, and pharmacotherapy as diagnostic and monitoring material (Giannobile et al., 2011). Saliva is now a highly popular diagnostic sample of choice due to: (i) its cost-effectiveness, bioavailability, non-invasive accessibility, and salivary analytes are relatively stable for storage (Sukriti et al., 2020), (ii) collection is fast, inexpensive and safe, and (iii) can be used to monitor and treat patients with both oral and systemic diseases. Saliva has been observed to be essential in the testing of many bioactive molecules. Of interest are previous studies that reported the assessment of some cytokines in saliva using different techniques(Hu et al., 2006; Pels, 2015; Slavish et al., 2015).

1.3.2 Urticaria Activity Score
CU treatment aims to attain significant improvements in symptoms with minimum adverse effects. Assessment tools have been designed and validated to help in the treatment of CU by evaluating the disease activity and its impact on the control and quality of life of CU. These include the angioedema activity score (AAS), urticaria and angioedema quality of life questionnaires (CU-Q2Ol, AE-QoL), the urticaria control test (UCT), and urticaria activity score (UAS) (Petkova & Staevska, 2020). The UAS was the only assessment tool
that was used in this study. The UAS tool is used to measure and monitor disease activity in chronic spontaneous urticaria as well as in some cases of patients with CINDU (Mathias et al., 2012). A version of the tool, Urticaria Activity Score 7 (UAS7) (Table 2) is the recommended approach for assessing treatment in CU (Beck et al., 2017; Engin et al., 2020; Mlynek, Zalewska-Janowska, et al., 2008; Zuberbier et al., 2018; Zuberbier et al., 2014).

The UAS7 is a unified and simple self-scoring system used by patients. It is based on the assessment of key urticaria symptoms, wheals, and pruritus and it is the sum score of seven consecutive days, with a maximum score of 42 (Table 2). Guidelines recommend the use of UAS7 in routine clinical practice and trials to determine disease activity and response to treatment (Beck et al., 2017; Zuberbier et al., 2018; Zuberbier et al., 2014).

<table>
<thead>
<tr>
<th>Score</th>
<th>Number of Hives</th>
<th>Hives Itch severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Mild (Less than 20 wheals/24hours)</td>
<td>Mild (Present but not annoying or troublesome)</td>
</tr>
<tr>
<td>2</td>
<td>Moderate (20-50 wheals/24hours)</td>
<td>Moderate (Troublesome but does not interfere with normal daily activity or sleep)</td>
</tr>
<tr>
<td>3</td>
<td>Intense (More than 50 wheals/24hours or large confluent areas of wheals)</td>
<td>Intense (Severe pruritus, which is sufficiently troublesome to interfere with normal daily activity or sleep)</td>
</tr>
</tbody>
</table>

Table 1: Urticaria Activity Score (UAS7). (Adapted from Zuberbier et al 2018).

1.3.3 Pharmacological and Biological Agents

Treatment of chronic urticaria patients includes the use of pharmacological and biological agents. Also, general avoidance measures are recommended as they are found to be helpful in the treatment of chronic urticaria. These include avoiding triggers or aggravating factors such as heat, stress, alcohol, and certain drugs such as aspirin (acetylsalicylic acid), NSAIDS, angiotensin-converting enzyme (ACE) inhibitors (particularly if the urticaria present with angioedema, with or without wheals), and codeine. Cooling, antipruritic
lotions such as calamine or 1% menthol in aqueous cream can be applied (Poonawalla & Kelly, 2009; Zuberbier et al., 2009).

### 1.3.4 H1-Antihistamines

For the pharmacological treatment of chronic urticaria see Table 3. The first line of therapy for symptomatic CU involves the administration of modern second-generation antihistamines (H1 receptor blocking agents) in licensed doses (Beck et al., 2017; Bulkhi et al., 2017; Curto-Barredo et al., 2014; Zuberbier et al., 2018). The first-line treatment achieves control in 40% of all CU patients (Kulthanan et al., 2008) and has a good safety profile. The use of first-generation antihistamines is now not recommended for the routine management of CU because of sedating side effects (Petkova & Staevska, 2020; Zuberbier et al., 2018).

![Recommended treatment algorithm for urticaria](image)

Table 2: Recommended treatment algorithm for urticaria. Adapted from Zuberbier et al., 2018. sgAH, secondary-generation H1-Antihistamine.

However, if the first-line treatment is ineffective, the second-line treatment which involves a gradual increase to up to fourfold the standard/conventional dose of the modern second-generation H1-antihistamines is recommended (Bernstein et al., 2014; Engin et al., 2020; Zuberbier et al., 2018). Second-line treatment is recommended in patients with CU with inadequate control or intolerable symptoms with 2-4 weeks of treatment or earlier (Staevska et al., 2010). 70% of CU patients achieve disease control with this dose (Kaplan,
CU patients who do not respond to this up-dosing are not recommended to receive further up-dosing but a third-line treatment. Third-line treatment includes adding on omalizumab to the non-sedating second-generation H1-antihistamines (Petkova & Staevska, 2020; Zuberbier et al., 2018).

Many studies have demonstrated that omalizumab (anti-IgE) is very effective and safe in the treatment of CSU and inducible urticaria (Boyce, 2006; Bulkhi et al., 2017; Giménez-Arnau et al., 2016; Kaplan et al., 2013; Maurer, Altrichter, et al., 2011; Maurer, Rosén, et al., 2013; Saini et al., 2011). Omalizumab prevents angioedema development, significantly improves the quality of life, and is suitable for long term treatment. The recommended dose is 300 mg every 4 weeks. Add on treatment with omalizumab is effective in 80-85% of patients with CU (Kaplan, 2018; Kocatürk et al., 2017). (Omalizumab details and mode of action are discussed in detail below). However, if there are patients with severe disease refractory to combinations of antihistamine and omalizumab a fourth-line treatment is recommended. The fourth-line treatment includes adding cyclosporin A to non-sedating second-generation H1-antihistamines (Kaplan, 2018; Mitchell et al., 2015) but is not recommended as standard treatment because of the higher risk of adverse effects on blood pressure and renal function (Kaplan, 2017). Cyclosporin A administration is known to increase the success rate of CU treatment by up to 93% (Kaplan, 2018). Besides using the conventional doses, patients with CINDU are advised to avoid known eliciting factors such as lifting heavy objects, intense pressure in delayed pressure urticaria, or friction in dermographism (Petkova & Staevska, 2020). Eradication of infection agents and treatment of inflammatory processes are recommended (Chiu et al., 2013; Wedi et al., 2004; Wedi et al., 2009). Reduction of physical and emotional stress is beneficial as there is some evidence that the disease activity and severity are correlated with stress levels (Varghese et al., 2016).

### 1.3.5 Omalizumab

Omalizumab is a recombinant human anti-IgE monoclonal antibody (Antia et al., 2018; Criado et al., 2015; Engin et al., 2020; Zuberbier et al., 2018). Omalizumab was originally approved for the treatment of allergic asthma (Antia et al., 2018).

It binds to and neutralises IgE hence preventing/impeding the IgE allogenic pathway (preventing IgE binding to the high affinity IgE receptor FCeR1) and render mast cells and basophils insensitive to activation through IgE/ FCeR1 (Chang & Shiung, 2006; Chang et
This process decreases the release of mediators responsible for the formation of pruritus and oedema. In CU patients, it is administered as monthly subcutaneous injections at doses of 300 mg (Criado et al., 2015; Engin et al., 2020; Zuberbier et al., 2018). The patient’s response to treatment is followed up after a six-month treatment. If the disease recurs, omalizumab treatment can be continued. It is an efficacious treatment alone or as an add-on therapy for CU cases that are resistant to high-dosage second-generation antihistamine treatment (Table 3). Omalizumab is known to be a safe treatment option and has favourable risk/benefit (Kaplan et al., 2013; Maurer, Rosén, et al., 2013; Saini et al., 2015). It has rarely been associated with anaphylaxis (Saini et al., 2015). Even though multiple biological drugs (rituximab and intravenous immunoglobulins) have been evaluated for use in antihistamine-refractory chronic urticaria patients, omalizumab is the most efficacious and is, therefore, the first licensed biological treatment available for chronic urticaria (Bulkhi et al., 2017; Giménez-Arnau et al., 2016). Several case reports and pilot studies conducted and recorded between 2006 and 2008 indicate favourable omalizumab efficacy against urticarial diseases (Boyce, 2006; Güzelbey et al., 2008; Kaplan et al., 2008; Metz et al., 2008; Sands et al., 2007; Spector & Tan, 2007). Additionally, there are clinical trials that convincingly established the efficacy and safety for treating CU patients with omalizumab that cannot be adequately treated with current standard care (Kaplan et al., 2013; Maurer, Altrichter, et al., 2011; Maurer, Rosén, et al., 2013; Saini et al., 2011). It is noteworthy to know that besides omalizumab, a short course of other drugs, such as cyclosporin Table 3, is also recommended in patients with refractory CU (Beck et al., 2017; Zuberbier et al., 2018; Zuberbier et al., 2014).

1.4 The Role of sTREM-1, IL-8, and IL-3 in CU

1.4.1 The Role of sTREM-1 in CU

The association of the soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1) with CU was investigated in this study. sTREM-1 is a small soluble protein synthesised by the alternative splicing of the Trem-1 gene or the proteolytic cleavage of the TREM-1 receptor protein by metalloproteinasies (MMPs) (Gómez-Piña et al., 2007; Palazzo, Simpson, Simmons, et al., 2012; Tammaro et al., 2017). In recent years, numerous authors have noted the essential role of sTREM-1 in bacterial and viral, parasitic, and fungal infections (discussed in Chapter 3). They have strongly suggested that it can be used as a marker of infection in adults and children (Garofoli, 2010).
Interestingly several other recent studies have presented evidence that sTREM-1 is raised in periodontal diseases (Bostanci & Belibasakis, 2012; Bostanci, Oztürk, et al., 2013; Bostanci et al., 2011; Nylund et al., 2018; Räisänen et al., 2020; Willi et al., 2014).

It is suspected that Porphyromonas gingivalis stimulates TREM-1 production in monocytes, promoting a shift from its cell-bound form to sTREM-1, resulting in increased levels of sTREM-1 which is then accompanied by propagation of pro-inflammatory cytokine production (Belibasakis et al., 2014). As far as it is known, there are currently no studies investigating sTREM-1 concentrations in the saliva of chronic urticaria patients. Defining this association would provide a valuable tool in identifying cases of chronic urticaria which may be associated with infection. The role of sTREM-1 in CU is described in more detail in Chapter 3.

Cytokines play a pivotal role in the host response to the bacterial challenge. A balanced production of cytokines results in the development of immunity (Bostanci et al., 2007). However, an imbalanced production of these cytokines (pro-inflammatory and regulatory cytokines) is suspected to cause tissue destruction and disease progression (Gemmell et al., 1997; Landi et al., 1997; Oido-Mori et al., 2001; Taylor et al., 2004).

1.4.2 The Role of IL-8 in CU

The association of IL-8 with chronic urticaria was also investigated in this study. Previous studies have shown that inflammatory cytokines including IL-8 are present in the diseased periodontal tissues and their uncontrolled production seems to play a role in tissue destruction (Bascones et al., 2005; Okada & Murakami, 1998). Several previous studies have provided evidence to support the concept that activated mast cells undergoing degranulation release some cytokines including IL-8 (Curto-Barredo et al., 2014; Zuberbier et al., 2014). Besides, other studies suggest TREM-1 enhances proinflammatory chemokine production (Belibasakis et al., 2014; Varanat et al., 2017). Kuai et al (2009) discovered that TREM-1 induces the production of multiple pro-inflammatory cytokines including IL-8. Wang et al (2012) reported that TREM-1 positively regulates the production of IL-8. Derive et al (2012) discovered that TREM-1 inhibition was linked to a decrease in the production of LPS- induced cytokines including IL-8. It is also speculated that microorganisms such as Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, and Aggregatibacter actinomycetemcomitans and the toxins they release in periodontal tissues stimulate the formation of IL-8 (Ertugrul et al., 2013; Mathur et al.,
1996; Tsai et al., 1995). As far as it is known, there are currently no studies investigating IL-8 concentrations in the saliva of chronic urticaria patients. Defining this association would provide a valuable tool in identifying cases of chronic idiopathic urticaria associated with infection.

The role of IL-8 in CU is described in more detail in Chapter 4.

1.4.3 The Role of IL-3 in CU

The association of IL-3 with chronic urticaria was also investigated in this study. As far as it can be ascertained there are currently no studies involving IL-3 in saliva. IL-3 is suspected to associate with the upregulation of CD203c as well as CD69 expression that results in basophil activation and histamine release. CD69 is a human transmembrane C-Type lectin protein encoded by the CD69 gene. It is an early activation marker that is expressed in hematopoietic stem cells, T cells, basophils (Vasagar et al., 2006), and many other cell types in the immune system.

CD203c upregulation is suggested to be mediated by either FceRIα or IL-3 with increased CD203c expression on basophils known to be associated with exacerbated chronic urticaria symptoms (Ye et al., 2014). CD69 is induced after basophil exposure to IL-3 and is thought to require de novo synthesis (Vasagar et al., 2006). An in vitro study by Zuberbier et al., (1996), showed that pre-treatment of basophils with interleukin IL-3 upregulated responsiveness of basophil histamine release (histamine release was 14.3%) to anti-IgE in urticaria patients. In a separate study by Ferrer et al., (2003) basophils primed with IL-3 had a histamine release of at least 15% more than those tested without priming. Autocrine IL-3 upregulation of mast cell survival in the absence of antigen has been reported by Shimizu et al (2008) as instrumental in the exacerbation of chronic urticaria symptoms. Published data indicate that IL-3 is released as a mediator by activated mast cells undergoing degranulation (Chang et al., 2015; Curto-Barredo et al., 2014; Zuberbier et al., 2014). The role of IL-3 in CU is described in more detail in Chapter 5.

1.5 Study Hypothesis, Aims and Objectives.

To date no study has investigated cytokines in the saliva of patients with CU. Therefore, this study will use enzyme-linked immunosorbent assay (ELISA) to measure the levels of sTREM-1, IL-8, and IL-3 in the saliva of healthy subject controls and patients with chronic urticaria.
1.5.1 Study Hypothesis
This study tests the hypothesis that there are significantly higher salivary sTREM-1, IL-8, and IL-3 levels in chronic urticaria patients compared to normal control subjects and that IL-8 levels are correlated with salivary sTREM-1 and are predictive of CU.

1.5.2 Study Aims.
This study aims to determine and compare the levels of sTREM-1, IL-8, and IL-3 in the saliva of healthy subject controls and patients with chronic urticaria, with a view to determining their utility as potential novel biomarkers of CU.

1.5.3 Study Objectives
- To obtain ethical approval for the collection of saliva samples from CU patients and healthy controls, 50 in each group
- To obtain written informed consent from patients and healthy controls
- To collect saliva samples from each group using the Salivette method
- To process saliva samples with protease inhibitors and store at -80°C for subsequent analysis of the cytokines sTREM-1, IL-8, and IL-3 by ELISA
- To validate commercial ELISA kits for analysis of cytokines in saliva
- To quantitatively analyse cytokines in patient and control saliva samples
- To carry out appropriate statistical analysis of the data to investigate differences between CU patients and healthy control salivary cytokine concentrations, and the predictive value for each cytokine in CU
Chapter 2: Method and Materials

Samples of saliva used in the study were collected from patients with CU over a period of 1 year at University Hospital Southampton (UHS), Southampton, United Kingdom. A diagnosis of CU in all patients was confirmed by the Immunologist Consultant, Dr Efrem Eren.

2.0 Ethics, Research & Development Approvals

Protocols for the study were approved by the Research Ethics Committee (REC), REC reference: 14/NI/1089, Health Research Authority England (HRA), and local Research and Development (R&D) R&D reference: RHM PAT0290. This study was conducted following the Research Governance Framework for Health and Social Care (2005) and Good Clinical Practice. Informed consent was obtained from all participants of the study.

| Table 3: CU patients, treatment-naïve, omalizumab treated and healthy controls |
|-----------------------------|-------------|-------------|-------------|-------------|
| NAIVE                      | Male | Female | Age 1-55 | CIU | CSU | +Angioedema |
| N=15                       | 3    | 12     | 21-55    | 1   | 14  | 8           |
| OMALIZUMAB TREATED         | Male | Female | Age 19-73| CIU | CSU | +Angioedema |
| N=28                       | 10   | 18     | 19-73    | 1   | 26  | 5           |
| CONTROLS                   | Male | Female | Age 22-57| CIU | CSU | +Angioedema |
| N=34                       | 18   | 16     | 22-57    | 0   | O   | O           |

Table 4: Total number of patients and healthy controls tested for sTREM-1, IL-8, and IL-3

| Table 4: Total number of patients and healthy controls tested for sTREM-1, IL-8, and IL-3 |
|-----------------------------|-------------|-------------|-------------|
| Patients                   | sTREM-1 | IL-8 | IL-3 |
|                           |        |     |     |
| Controls                   | 43     | 40  | 43  |
|                           | 34     | 31  | 30  |

2.1 Patients and Healthy Controls Selection

This was a pilot case-control study. The total number of participants included in the study amounted to 77 (Tables 3 and 4). Forty-three were patients with chronic urticaria, with or without angioedema, of which thirteen were male and thirty were female. Thirty-four normal controls were also recruited, with eighteen being male and sixteen being females.
The age range for all participants was 21-73 years for patients and 22-57 years for the healthy controls. Total chronic urticaria patients were made up of 70% females and 30% for males. The total percentages per gender of the total control sample were 47% females and 53% for males. All participants (patients and controls) invited to participate in the study were volunteers who were randomly picked from patients visiting the immunology and allergy clinic at University Hospital Southampton (UHS).

All patient and control samples were fully anonymised and no patient identifiable information such as name and hospital number were available. Each sample was given a unique identifier number with no other information.

The purpose of the study and associated procedures were fully explained to all participants, and it was clarified that participation was voluntary. Before enrolment, informed written consent was obtained from all individuals who agreed to participate in the study (Appendix B and C), and all participants were given the patient information sheet to read (Appendix A). Of the total patients, 63% were on Omalizumab treatment whilst 37% were naïve (untreated).

The medical history of the patients was obtained through the hospital’s electronic records by the consultant immunologist. The healthy controls had no known history of chronic urticaria. Patients below the age of 18 and above 80, and any patient reporting their use of aspirin or any NSAID on the day the samples were collected were excluded from this study. Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are known to exacerbate the pre-existing chronic spontaneous urticaria as explained in Chapter 1.

A further 10 healthy control volunteers (UHS staff) were included for salivary TREM-1 and IL-3 assay validation and optimization before sample processing.

The UAS7 was used to score urticaria symptoms for CU patients as shown in Table 5. The UAS7 is a unified and simple self-scoring system used by patients. It is based on the assessment of key urticaria symptoms, wheals, and pruritus and it is the sum score of seven consecutive days, with a maximum score of 42. It is used in routine clinical practice and trials to determine disease activity and response to treatment (Beck et al., 2017; Zuberbier et al., 2018; Zuberbier et al., 2014).
<table>
<thead>
<tr>
<th>Date</th>
<th>Daily number of wheals (0-3)</th>
<th>+</th>
<th>Daily intensity of pruritus (0-3)</th>
<th>=</th>
<th>Daily UAS score (0-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example</td>
<td>0 1 2 3</td>
<td>+</td>
<td>0 1 2 3</td>
<td>=</td>
<td>0 1 2 3 4 5 6</td>
</tr>
<tr>
<td>1</td>
<td>0 1 2 3</td>
<td>+</td>
<td>0 1 2 3</td>
<td>=</td>
<td>0 1 2 3 4 5 6</td>
</tr>
<tr>
<td>2</td>
<td>0 1 2 3</td>
<td>+</td>
<td>0 1 2 3</td>
<td>=</td>
<td>0 1 2 3 4 5 6</td>
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<td>3</td>
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<td>+</td>
<td>0 1 2 3</td>
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<td>4</td>
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<td>0 1 2 3</td>
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<td>0 1 2 3</td>
<td>+</td>
<td>0 1 2 3</td>
<td>=</td>
<td>0 1 2 3 4 5 6</td>
</tr>
</tbody>
</table>

Table 5: Patients’ UAS7 scoring sheet

### 2.2 Saliva Sample Collection and Processing

Saliva samples were collected from patients and controls with written informed consent. These samples were collected and analysed adhering to protocols submitted to REC and HRA. The method of collection was extremely hygienic, using screw cap Salivette® system (Sarstedt combined pot and biocompatible synthetic swab) Figure 9

![Salivette collection system](image)

**Figure 9:** Salivette collection system – together and in parts. The saliva-soaked swab (B) is put into C (the insert) and then the insert is put into the D (the tube), which is then closed by the lid cap (A, the stopper)

The saliva sample collection performed in this study is a modification of protocols as described by the Sarstedt website and previously published methods (Esser et al., 2008; Thomadaki et al., 2011). Prior to sample collection, participants were asked to avoid flossing, brushing, mouth rinses, eating, and drinking for 2 hrs. Participants were asked to
rinse their mouths with tap water and asked to insert the pad (absorbent swab) into the mouth, either under the tongue or in the cheek, or to chew it. The absorbent swab was kept in the mouth to soak up saliva for a standardized period of 2 minutes. The saliva-soaked swab was transferred to a storage container and placed on ice until the sample was centrifuged on the same day of collection.

The samples were centrifuged at 3000 rpm (1500g) for 15 minutes at 4º C. The resultant supernatants (1 mL) were immediately aliquoted into sterile tubes, supplemented with 10µl of EDTA- Protease Inhibitor Cocktail -Calbiochem (Merk life Science UK Limited, Dorset, United Kingdom) using sterile tips and immediately frozen at low temperature in an “ultra-cool temperature freezer” at -80ºC to prevent microbial growth and avoid degeneration of sTREM-1 and the cytokines (IL-3 and IL-8). Further analysis of the saliva samples was performed using the ELISA technique Figure 10.

All pipetting and sample preparation were conducted under sterile conditions. Reagents preparations, sample pipetting, the addition of detection antibody were performed in a sterile fume hood. Distilled water used to make up reagents was filtered, and sterile containers were always used.

### 2.2.1 Enzyme-linked Immunosorbent assay (ELISA)

![Diagram of ELISA process]

Figure 10: A sandwich enzyme-linked immunosorbent assay
Step 1; Capture antibody is bound to the wells of a 96-well plate. Unbound antibody is washed away.
Step 2; Sample with antigen of interest is added at an appropriate dilution to be measured against a standard curve prepared with isolated antigen
Step 3; HRP-labelled detection antibody is added
Step 4; The HRP substrate TMB is added
Step 5; Coloured product is measured using a plate reader
The ELISA test explores the principle of antibody-antigen reaction to identify and quantify substances (Lugos et al., 2019) such as antigens, cytokines, antibodies, glycoproteins, and proteins in biological samples (Raditic et al., 2011; Woodbury et al., 2002). The ELISA technique comprises three main analytical approaches, direct ELISA, indirect ELISA, and sandwich ELISA (Crowther, 2000). A sandwich enzyme-linked immunosorbent assay (ELISA) was the test technique used in the salivary sTREM-1, IL-8, and IL-3 experiments Figure 10. The sandwich ELISA was preferred over the other two approaches because it can bind the target antigen in impure samples selectively and also has a higher specificity since the antibodies used are against different epitopes of the target antigen (Osmekhina et al., 2010). The sample value obtained from an ELISA is dependent upon the interaction between the protein of interest and the ELISA’s antibodies, and a comparison of this interaction relative to a recombinant protein standard curve. ELISA signal generation is influenced by several factors but in this study, the focus was on variable characteristics related to the target antigen (IL-3, IL-8, and sTREM-1) such as optimum dilutions and sample matrix.

Dilutions, sample matrix, buffer components, complement, heterophilic antibodies, and rheumatoid factor are known to have a marked effect on the accuracy of ELISA results (Bratcher & Gaggar, 2014; R & D Systems, 2006). It was therefore imperative to perform spike/recovery and linearity experiments on saliva samples to determine whether values reported from previously unvalidated saliva sample types were accurate. The R & D systems (2006) method from Thermo Fisher Scientific (2007) protocols and a few modifications from (Andreasson et al., 2015) were adopted.

The Quantikine Human IL-3 and the DuoSet Human (TREM-1and IL-8) Immunoassay kits (R&D Systems) are validated to analyse IL-3, TREM-1, and IL-8 in cell culture supernatants, serum, and plasma, respectively. They are not validated to analyse salivary IL-3, sTREM-1, and IL-8.

Saliva samples were spiked with a known amount of ELISA standard. The amount recovered was compared to an identical amount in a standard diluent. Salivary sTREM-1 and salivary IL-3 validation experiments (Spike/Recovery and Linearity experiments) are described in detail in below. There were no Spike/Recovery and Linearity experiments performed for salivary IL-8 due to time constraints. It is assumed therefore that recovery of
IL-8 in saliva is the same as for the more complex matrix represented by serum and plasma and is also in the acceptable recovery range of 80-120%.

2.2.2 Spike, Recovery, and Linearity Experiments by ELISA.
Spike/recovery of an analyte assesses the ability of an assay to detect a known concentration of analyte added to a matrix under investigation. It determines whether the detection is affected by the biological sample matrix compared with the standard curve diluent (Lee et al., 2006). Spike/recovery is essential for the analysis and accuracy evaluation of the ELISA method for particular sample types. Preferably the % recovery should not be less than 100% but 80-120% is considered satisfactory (Andreasson et al., 2015; R&D Systems, 2006).

A known amount of the standard is added to a sample and the resulting concentration of the spiked material indicates whether a component in the sample interferes with the ELISA. Linearity with dilution is performed to demonstrate the ability to detect an analyte that has been diluted to concentrations within the standard curve without affecting accuracy and precision.

2.2.3 Sample and Control Spike/Recovery Preparation
Calculations for the spike/recovery were performed using the formula below:

\[
\% \text{ Recovery} = \frac{\text{Observed} - \text{Neat}}{\text{Expected}} \times 100
\]

Observed = Spiked sample value
Neat = Unspiked sample value
Expected = Amount spiked into the sample (calculated based on the assigned concentration of spiking stock and volume spiked into sample).
The acceptable recovery range was 80-120%.

2.2.4 Linearity
Spike control, spike sample, and neat sample were serially diluted 1:2, 1:4, 1:8 and tested to assess linearity. Calculations for linearity recovery were performed using the formula below:

\[
\% \text{ Recovery (1:n)} = \frac{\text{Observed value (pg/mL) of 1:n dilution}}{\frac{\text{Expected value (pg/mL) divided by n}}{\text{Expected value (pg/mL) divided by n}}} \times 100
\]

n = 2, 4 or 8.
Spiked sample and spiked control values were used as expected values for testing the linearity of the spiked sample and spiked control, respectively.

The neat sample value was used as the expected value for testing the linearity of the neat sample.

The acceptable recovery range was 80-120%.

**2.3 Soluble TREM-1 Spike/Recovery and Linearity Experiments.**

The DuoSet Human TREM-1 Immunoassay kit; Catalog# DY1278B (R&D Systems) was the kit used in this study as it able to detect soluble TREM-1. This kit is validated to analyse soluble TREM-1 in cell culture supernatants, serum, and plasma but not validated to analyse salivary soluble TREM-1. It was therefore imperative to perform spike/recovery and linearity experiments on saliva samples to determine whether values reported from previously unvalidated saliva sample types were accurate. A method provided by the commercial supplier of the ELISA kit (R & D systems) was used to check the impact of the sample matrix on results reported from unvalidated saliva samples. The validated assay was applied to measure total salivary soluble TREM-1 in chronic urticaria patients and compared to levels obtained in apparently healthy controls.

**2.3.1 TREM-1 Standards Calculations**

Two standard stock solutions with different concentrations (180,000 pg/ml and 140,000 pg/ml) of recombinant human TREM-1, were used (R&D Systems). Running out of the first batch of reagents whose standard stock solution was 180,000 pg/ml necessitated the change of the standard stock solution to 140,000 pg/ml. The expected concentrations of the spike control and spike sample used were 3600 pg/ml and 2800 pg/ml as calculated below.

\[
\text{20µl of standard + 980mls of reagent diluent or sample} \\
\text{This dilution} = \frac{20}{1000} = \frac{1}{50} \\
\frac{1}{50} \text{ of 180,000 pg/ml} = 3,600 \text{ pg/ml} \\
\frac{1}{50} \text{ of 140,000 pg/ml} = 2,800 \text{ pg/ml}
\]
Thus, 3,600 pg/ml and 2,800 pg/ml were the final concentrations of sTREM-1 in the control (diluent) and control saliva (healthy donor) sample.

2.3.2 TREM-1 Sample, Control Spike and Linearity Preparations

As far as it is known there is no previous evidence/data of spike/recovery and linearity experiments quantifying sTREM-1 in saliva. Validation for saliva sample matrix was performed using standards (recombinant human TREM-1) provided in the kit and samples from normal controls (healthy donors). The highest standard concentration of the appropriate recombinant human TREM-1 was 6000 pg/ml. This was further serially diluted to prepare the standard curve. The TREM-1 DuoSet kit had no premade commercial controls.

Three tubes were labelled, neat, spike sample, and spike control. From a well-mixed saliva sample (healthy donor saliva sample- 4 different healthy donor samples were used), 1000μl of healthy control saliva sample was added to a tube as the unspiked- neat sample and 980μl were pipetted into the tube labelled spike sample. 980μl of reagent diluent was pipetted into the tube labelled spike control. 20μl of the TREM-1 spiking stock solution was added into the spike sample tube to prepare the spike sample. 20 μl of TREM-1 stock solution was added to 980 μl of diluent to prepare the control spike.

For linearity, serial dilutions (1:2, 1:4, 1:8) of the 3600 pg/ml and 2800 pg/ml concentrate were performed with diluent for both spike control and spike sample respectively. The starting 3600 pg/ml concentration gave expected values of 1800 pg/ml, 900 pg/ml, and 450 pg/ml spike. The starting 2800 pg/ml concentration gave expected values of 1400 pg/ml, 700 pg/ml, and 350 pg/ml spike. Calculations for the spike/recovery and linearity were performed using the formula given above.

2.3.3 Assay Procedure for Soluble TREM-1

Estimation of sTREM-1 was performed by an enzyme immunoabsorbent assay using a DuoSet kit manufactured by R & D systems and used according to the manufacturer’s instructions. Capture antibody (goat anti-human TREM-1) was reconstituted in PBS and was diluted to 800 ng/mL (0.8 μg/ml) in PBS for distribution in a 96-well plate at a volume of 100 μL per well and incubated overnight at room temperature. After overnight incubation, wells were washed three times with a 0.05% solution of Tween 20 in PBS (pH: 7.2–7.4). 300 μL of blocking buffer (5% Tween 20 in PBS, Ph 7.2-7.4, 0.2 μm filtered)
was added to each well and incubated for 1 hour at room temperature. After 1-hour incubation the wells were washed three times with 200 µL of a 0.05% solution of Tween 20 in PBS. Then 100 µL of standard concentrations of TREM-1 (95–6000 pg/mL, R&D Systems) serially diluted in reagent diluent (5% Tween 20 in PBS, pH 7.2-7.4, 0.2µm filtered), with reagent diluent as the blank or saliva was added in wells and incubated at room temperature for 2 hours. After incubation for 2 hours, wells were washed three times, and 100 µL of a 400 ng/mL dilution of the TREM-1 detection antibody (biotinylated goat anti-human TREM-1 detection antibody) in reagent diluent (with 2% normal goat serum) was added per well. The plate was incubated for 2 hours at room temperature and after incubation wells were washed three times. 100µl of the working concentration of streptavidin–horseradish peroxidase was added to each well to bind to and label the attached antibodies and incubated for 20 minutes at room temperature in the dark. After incubation, the wells were washed three times. 100 µl of a freshly prepared substrate solution (equal parts of colour reagent A (hydrogen peroxide) + colour reagent B (tetramethylbenzidine) was added to each well and incubated for 20 minutes at room temperature in the dark. 50 µl of stop solution (2N sulphuric acid, R&D systems) was added to each well with resultant colour change from blue to yellow proportional to TREM-1 quantity. All determinations were performed in duplicate. The absorbance was estimated using a microplate reader set at 490nm minus reference wavelength correction at 650nm with SoftMax PRO software.

2.4 IL-8 Spike, Recovery, and Linearity Experiments
There were no Spike/Recovery and Linearity experiments performed for salivary IL-8. IL-8 was one of the first cytokines to be measured by ELISA. There are many studies that have found recovery of IL-8 from complex matrices like plasma and serum already between 80-120% and therefore in a simple matrix like saliva the recovery of IL-8 is likely to be as good as recovery from plasma and serum. It is assumed therefore that recovery of IL-8 in saliva is the same as for the more complex matrix represented by serum and plasma, and is in the acceptable recovery range of 80-120%.

2.4.1 Assay Procedure for IL-8.
Estimation of IL-8 was performed by an enzyme immunoabsorbent assay using a DuoSet (Catalog# DY208) kit manufactured by R & D systems and used according to the manufacturer’s instructions. Capture antibody (mouse anti-human IL-8) was reconstituted
in PBS and diluted to 4000 ng/mL in PBS for distribution in a 96-well plate at a volume of 100 µL per well and incubated overnight at room temperature. After overnight incubation, wells were washed three times with 200 µL of a 0.05% solution of Tween 20 in PBS (pH: 7.2–7.4). Blocking buffer (1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered) was added to each well and incubated for 1 hour at room temperature. After 1-hour incubation the wells were washed three times with 200 µL of a 0.05% solution of Tween 20 in PBS. Then 100 µL of standard concentrations of IL-8 (15.625–1000 pg/mL, R&D Systems) serially diluted in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.2-7.4, 0.2 µm filtered), with reagent diluent as the blank, or neat saliva was added in wells and incubated at room temperature for 2 hours. After incubation for 2 hours, wells were washed three times, and 100 µL of a 20 ng/mL dilution of the IL-8 detection antibody (biotinylated goat anti-human IL-8 detection antibody) in reagent diluent was added per well.

The plate was incubated for 2 hours at room temperature and after incubation wells were washed three times. 100µl of the working concentration of streptavidin–horseradish peroxidase was added to each well to bind to and label the attached antibodies and incubated for 20 minutes at room temperature in the dark. After incubation, the wells were washed three times.

100 µl of a freshly prepared substrate solution (12 ml ELISA Substrate Buffer (0.11 M acetate buffer, pH 5.5), 200µl TMB 3,5,3’,5’-tetramethylbenzidine, 1.2µl 30% H₂O₂) was added to each well and incubated for 15 minutes at room temperature in the dark. 100 µl of stop solution (2M sulphuric acid) was added to each well with resultant colour change from blue to yellow proportional to cytokine quantity. All determinations were performed in duplicate. The absorbance was estimated using a microplate Dynex Technology plate reader set at 450nm.

2.5 IL-3 Spike, Recovery, and Linearity Experiments

In this study, IL-3 is detected using the Quantikine Human IL-3 Immunoassay sandwich ELISA kit; Catalog# D3000 (R&D Systems, 2017). However, this kit is optimised for detecting IL-3 in cell culture supernate, serum, and plasma (R&D Systems, 2017). It is not validated for salivary IL-3 estimation. A sandwich ELISA kit (R & D systems) built on commercially available reagents was used to check the impact of the sample matrix factors
on results reported from unvalidated saliva samples. The validated assay was applied to measure total salivary IL-3 in chronic urticaria patients and compared to levels obtained in apparently healthy controls.

2.5.1 Sample and Control Spike Preparation for IL-3
As far as it is known there is no previous evidence/data of spike/recovery and linearity of quantifying IL-3 in saliva. Standards were provided in the kit, and samples from healthy donor controls were used. The standard stock solution concentration for IL-3 was 20,000pg/ml (R&D Systems). This was prepared by diluting 10ng of lyophilised recombinant human IL-3 in buffered protein with 500μl calibrator/diluent (R&D systems).

Three tubes were labelled, neat, spike sample, and spike control. From a well-mixed saliva sample, 1000μl were pipetted into the tube labelled neat and 980μl were pipetted into the tube labelled spike sample. 980μl of reagent diluent was pipetted into the tube labelled spike control. 20μl of spiking stock solution (20,000 pg/ml) was added into the spike sample tube and spike control tube. 6 different healthy donor control saliva samples were used. The expected concentration of the spike control and spike sample was 400pg/ml as calculated below.

20μl of standard + 980mls of reagent diluent or sample

\[
\text{This dilution} = \frac{20}{1000} = \frac{1}{50}
\]

\[
\frac{1}{50} \text{ of 20,000 pg/ml} = 400 \text{ pg/ml}
\]

Thus, 400 pg/ml was the final concentration of IL-3 in the control (calibrator diluent) and control saliva (healthy donor) samples.

Calculations for the spike/recovery were performed using the formula given above.

For linearity serial dilutions (1:2, 1:4, 1:8) of the 400 pg/ml concentrate were performed 5 times with diluent for both spike control and spike sample. The starting 400 pg/ml concentration gave expected values of 200 pg/ml, 100 pg/ml, and 50 pg/ml spike. The highest concentration of the appropriate recombinant IL-3 standard was 2000 pg/ml. This was further serially diluted to prepare the standard curve.
2.5.2 Quality Control Materials for IL-3
Three premade commercial quality control (QC) samples were also used to assess the assay precision. These were lyophilised recombinant human IL-3 Immunoassay control samples at low, medium, and high concentrations. They were reconstituted with distilled water (R&D-Bio-techne). They were analysed three times on one plate to assess intra assay precision and four times in separate ELISA assays to assay inter-assay precision.

Acceptable ranges for cell supernates were used: low:128-176pg/ml; medium: 453-575pg/ml; high:962-1174pg/ml +/- 3SD. The observed QC results of the salivary IL-3 assay were compared to the target values given (Tables 33 & 34).

2.5.3 Assay Procedure for IL-3
Estimation of IL-3 was performed by a solid phase enzyme immunoabsorbent assay using a Quantikine kit (R & D Systems) and used according to the manufacturer’s instructions with minimum modification. A microplate precoated with a monoclonal antibody specific for human IL-3 was used. 200 µL of standard concentrations of IL-3 (31.3-2000pg/ml, R & D systems) serially diluted in calibrator diluent, with calibrator diluent as the blank, or saliva was added in wells and incubated for 2 hrs at cold temperature(2-8ºC). After 2 hours of incubation, wells were washed three times with 200 µL of a 0.05% solution of Tween 20 in PBS, pH 7.2-7.4. 200 µL of an enzyme linked polyclonal antibody specific for human IL-3 was added (Human IL-3 conjugated to horseradish peroxidase) and incubated for two hours at 2-8 ° C. After incubation wells were washed three times. 200 µL of a freshly prepared substrate solution (equal parts of colour reagent A (hydrogen peroxide) + colour reagent B(tetramethylbenzidine) was added and incubated for 20minutes in the dark. 50 µL of stop solution (2N sulphuric acid, R&D systems) was added with resultant colour change from blue to yellow proportional to IL-3 quantity. All determinations were performed in duplicate. The absorbance was estimated using a microplate reader set at 490nm minus reference wavelength correction at 650nm with SoftMax PRO software.

2.6 Routine Blood Tests Assays
CRP, Vitamin D, C3 and C4, and ESR were analysed using the automated methods in UHS Biochemistry and Haematology Laboratory according to the standard operation procedures (SOPs). The researcher did not do these tests but got the results linked to these patients through the password protected laboratory system.
2.6.1 C-reactive protein (CRP) Assay

CRP was analysed on the AU5800 and AU 680 Beckman Coulter analysers (Beckman Coulter United Kingdom Ltd, High Wycombe, UK). The assay used is an Immuno-turbidimetric test (particle enhanced immunoturbidimetric test). The principle of the test is based on particles coated with the antibodies of interest, forming complexes with the antigen in the sample. Particle enhanced immunoturbidimetric tests are useful if an antigen is present in a low concentration in the sample. For the quantitative determination of CRP in human serum/plasma, the sample is mixed with buffer and antiseraum solution. The human CRP reacts specifically with anti-human CRP antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the CRP concentration in the sample. The normal sensitivity application for this test is 0.2-480 mg/L and the high sensitivity application is 0.08-80 mg/l. The normal ranges are age dependent:

Up to 1 year 0 – 3.6 mg/L
Greater than 1 year 0 – 7.5 mg/l.

2.6.2 Vitamin D Assay

Vitamin D was analysed on the DXI 800 Beckman Coulter analyser (Beckman Coulter United Kingdom Ltd, High Wycombe, UK). The 25 (OH) Vitamin D Total assay is a two-step competitive binding immunoenzymatic assay with two incubation steps. In the initial incubation, the sample is added to a reaction vessel with a Vitamin D binding protein (DBP) releasing agent and paramagnetic particles coated with sheep monoclonal anti-25 (OH) vitamin D antibody. 25 (OH) vitamin D is released from DBP and binds to the immobilized monoclonal anti-25 (OH) Vitamin D on the solid phase. Subsequently, a 25 (OH) vitamin D analogue-alkaline phosphatase conjugate is added which competes for binding to the immobilized monoclonal anti-25 (OH) vitamin D. After a second incubation, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate Lumi-Phos* 530 is added to the vessel, and the light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of 25 (OH) vitamin D in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve. The range of sensitivity of this test is 5-525 nmol/l. The reference ranges are as follows: <30nmol/L: Consistent with deficiency
30-50nmol/L: May indicate a deficiency
>50nmol/L: Adequate level

>374nmol/L: Toxicity possible

2.6.3 Complement C (C3) and Complement 4 (C4) Assay

C3 and C4 were analysed on the AU5800 Beckman Coulter analysers (Beckman Coulter United Kingdom Ltd, High Wycombe, UK).

The assay used is an Immuno-turbidimetric test (particle enhanced immunoturbidimetric test). The principle of the test is based on particles coated with the antibodies of interest, forming complexes with the antigen in the sample. Particle-enhanced immunoturbidimetric tests are useful if an antigen is present in a low concentration in the sample. For the quantitative determination of C4 (Complement 4) in human serum/plasma, the sample is mixed with buffer and antiserum solution. The human C4 reacts specifically with anti-human C4 antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the C4 concentration in the sample. For the quantitative determination of C3 (Complement 3) in human serum/plasma, the sample is mixed with buffer and antiserum solution. The human C3 reacts specifically with anti-human C3 antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the C3 concentration in the sample. The assay method was extracted from Automated Biochemistry Laboratory SOP. The lowest detectable level in serum for C3 is 0.0006 g/L. The reference range is 0.75–80 g/l. The lowest detectable level in serum for C4 is 0.001 g/l. The reference range is 0.14 – 0.54 g/l.

2.6.4 Erythrocyte Sedimentation Rate (ESR) Assay

The determination of the Erythrocyte Sedimentation Rate of whole blood samples was done on the StaRRsed RS (auto-compact) Analyser (Mechatronics Manufacturing B.V, Zwaag, The Netherlands) in Haematology. Blood samples for ESR were collected in potassium – ethylenediaminetetraacetic acid (K_2-EDTA) anticoagulated tubes.

StaRRsed analysis is based on the Westergren sedimentation technique with slight modifications. 3 ml of K_2-EDTA blood is taken for the ESR determination. The instrument uses a vacuum pump to aspirate 1.6 ml of blood sample and dilutes it with 0.4 ml of 3.8 % (105 mM) Na_3-Citrate solution. The diluted sample is then aspirated to the Westergren pipette, and the sedimentation is measured using the optical density at 950 nm after exactly 30 min. A correlation curve is then used to transform the results into 60 min
measurement time. The results are given in mm/h at 18°C using the temperature correction equation in the instrument according to the manufacturer.

The rate of sedimentation depends on the difference in specific gravity between red cells and plasma, and the ability of red cells to repel one another due to their negative surface charge. The assay method was extracted from Automated Haematology SOP and (Horsti et al., 2010).

ESR is measured in mm/1st hour. The reference ranges are age and gender dependant;

<table>
<thead>
<tr>
<th>Age</th>
<th>ESR-Male</th>
<th>ESR-Female</th>
</tr>
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<tbody>
<tr>
<td>Up to 10 years</td>
<td>10 mm</td>
<td>19 mm</td>
</tr>
<tr>
<td>51-60 years</td>
<td>12 mm</td>
<td>19 mm</td>
</tr>
<tr>
<td>61-70 years</td>
<td>14 mm</td>
<td>20 mm</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>30 mm</td>
<td>35 mm</td>
</tr>
</tbody>
</table>

2.7 Statistical Analysis

The Shapiro-Wilk test was used first to test whether the results were normally distributed. Mann-Whitney U test and the one-way ANOVA (Kruskal-Wallis test, with Dunns multiple comparisons) were used to assess the control-patient group comparisons, and p<0.05 values were considered statistically significant. Correlations among all assays were analysed by the Spearman’s rank test, and p<0.01 values were considered as significant.

ROC analysis was used to determine the predictive value of each cytokine for CU.

Statistical analysis was performed using the GraphPad Prism software version 8.0.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com)

2.7.1 Statistical analysis of salivary sTREM-1 and IL-3

Many of the results for salivary IL-3 and sTREM-1 lie below the level of detection (LOD) of the ELISA kits used for analysis. The LOD is the lowest analyte concentration likely to be reliably detected above the level of the blank (Armbruster & Pry, 2008). The LOD for the R&D Systems DuoSet ELISA to measure sTREM-1 according to the manufacturer’s instructions, has been reported as 23.2 pg/ml (Hasibeder et al., 2015) and for IL-3 measured using the R&D Systems Quantikine ELISA is reported to be 7.4 pg/ml (from the product insert).
2.7.1.1 Fisher Exact Test
In the first statistical analysis of the data, the Fisher Exact Test was applied to compare the proportions of samples from healthy controls and CU patients (untreated and treated with Omalizumab) with detectable or undetectable levels of sTREM-1 and IL-3.

2.7.1.2 Censoring the sTREM-1 and IL-3 data
In statistical analysis, values below the LOD may be censored and substituted with a constant value. There are many ways to substitute the value for analysis when the response is below the LOD threshold. Some investigators use zero, other investigators use the LOD itself, and still others split the difference and use half of the LOD, or the LOD/(sqrt2). Finally, some analysts regard such a case as indeterminate and so leave the value missing. These ad hoc approaches unfortunately introduce bias in any estimates. A missing value reduces the sample size and, therefore, the power of the analysis, as if nothing is known about the response when, in fact, there is information available. Using zero biases the estimates downward, using the LOD biases the estimates upward. Some analysts suggested that using half the LOD or LOD/(sqrt2) might average out the bias (Bailey & Michelson, 2013). Additionally some analysts proposed that substitution of values < LOD with LOD/(sqrt2), would not affect the relative differences between means when 50-60% of values were censored (Croghan & Egeghy, 2003). Therefore, in the data sets being analysed, values of IL-3 < LOD were substituted with LOD/(sqrt2) or 7.4/1.414 = 5.23 pg/ml. For sTREM-1 the substituted value was 23.2/1.414= 16.41 pg/ml.

These censored data sets were non-normally distributed and were analysed for differences between two groups using the Mann-Whitney test. Differences between healthy and untreated or OMA-treated groups were analysed using the Kruskal-Wallis ANOVA with Dunn’s multiple comparisons post-hoc test. To assess the performance of salivary sTREM-1, IL-8 and IL-3 analysis as diagnostic tests for CU, a receiver operating characteristic (ROC) curve over the range of possible cutpoints for the predictor variable was prepared for each analyte as described before (Mandrekar, 2010a, 2010b). The ROC curve indicates the sensitivity and specificity of the results by comparing all the results in a group of patients with a group of controls. Hence, the predictive value of a test was made using ROC analysis of the data from healthy controls and treated and/or untreated CU patients.
Chapter 3: Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1)

3.1 Triggering Receptors Expressed on Myeloid Cells Proteins

Triggering receptors expressed on myeloid cells (TREMs) are members of the immunoglobulin superfamily of cell surface receptors that participate in various cellular processes such as inflammation, coagulation, and bone homeostasis (Gómez-Piña et al., 2007). There are five members of the TREM family of proteins, namely Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1), Triggering Receptor Expressed on Myeloid Cells-2 (TREM-2), Triggering Receptor Expressed on Myeloid Cells-3 (TREM-3, a pseudogene in humans, but functional gene in mice) and homologous genes TREM-like transcript 1 (TLT-1) and TREM-like transcript 2 (TLT-2) (Bellingan et al., 2002; Pelham et al., 2014). The murine and human TREM genes cluster closely (Pelham et al., 2014). The TREM proteins are highly preserved in evolution and can also be found in chickens, pigs, and cows (Rudick et al., 2017).

TREM-1, a founding member of this family, is an activating and most studied receptor (Tammaro et al., 2017). It is a transmembrane receptor (Willi et al., 2014) identified in the last two decades (Bouchon et al., 2000; Bouchon et al., 2001) and it is located on the human chromosome 6p2 (Pelham et al., 2014). TREM-1 consists of an extracellular domain, a transmembrane region, and a short cytoplasmic domain that lacks signalling motifs (Colonna & Facchetti, 2003; Varanat et al., 2017). Hence for downstream signal transduction, TREM-1 requires the formation of an intracellular complex with Dnax activating protein 12 (DAP12) (Ormsby, 2011; Rudick et al., 2017; Tammaro et al., 2017) (Figure11). DAP12 is a transmembrane activating enzyme with an immunoreceptor tyrosine-based activation motif signalling unit that activates proinflammatory immune response (Colonna & Facchetti, 2003; Dong et al., 2017).
Figure 11: TREM-1 pathway.

The triggering receptor expressed on myeloid cells-1 (TREM-1), is a transmembrane receptor observed on the cell surface. It has an extracellular part, the Immunoglobulin-like (Ig) domain that binds to the ligand, and the transmembrane (TM) domain that binds to the adapter molecule DAP12. The TREM-1/DAP12 association causes the ITAM motifs of DAP12 to be phosphorylated by Src kinases. This then causes the recruitment and the binding of the spleen tyrosine kinase (SYK) and zeta-chain-associated protein kinase 70 (ZAP70). The SYK/ZAP70 activates Phosphoinositide phospholipase C gamma (PLC-γ), Phosphoinositide 3-kinases (PI3K), Janus kinase (JAK), and Mitogen-Activated Protein Kinases (MAPK) pathways. These pathways regulate Ca2+ influx, cell survival, and the upstream regulators of inflammatory gene transcription. The inflammatory responses are also triggered by other innate receptors such as Toll-like (TLRs), RIG-I-like (RLRs), and NOD-like receptors (NLRs). The membrane bound (mTREM-1) is cleaved at the cell surface by matrix metalloproteinases (MMP), leading to the formation of the soluble form (sTREM-1). The signalling pathways of the different pattern recognition receptors (PRRs) may also be amplified by TREM-1 as shown by segmented dotted lines in Figure 11. DAMPs: danger-associated molecular patterns; MAMPs: microbe-associated molecular patterns. (Adapted from de Oliveira Matos et al (2020).)

3.2 The Triggering Receptor Expressed on Myeloid Cells-1 Pathway

It is important to note that TREM-1/TLR pathway association results in interleukin-1 receptor associated kinase (IRAK1) that leads to the production of pro-inflammatory cytokines (TNF, IL-1α, IL-6, IL-8) through the NF-kB pathway (Fortin et al., 2007). The TREM-1/TLR pathway also dictates the degranulation of neutrophils. Phagocytosis, respiratory burst, and large production of proinflammatory cytokines are other outcomes of TREM-1 activation (Pandupuspitasari et al., 2016). TREM-1 activation also causes upregulation of other cell surface proteins such as CD11, CD29, CD80 (Bouchon et al., 2000; Fortin et al., 2007). When pathogens enter an organism they are recognised by PRRs.
(Khan et al., 2016). Neutrophils and monocytes express these PRRs which interact with microorganisms during bacterial invasion (Medzhitov & Janeway, 2000). TREM-1 is known to play a role in modulating PRR signalling by up or down regulation (Genua et al., 2014). Hence TREM-1 plays an active role as an amplifier of inflammation and is upregulated in response to lipopolysaccharide (LPS) (Bouchon et al., 2001).

3.3 The Triggering Receptor Expressed on Myeloid Cells-1 and Infections
The first experiments showed that TREM-1 is mainly expressed on neutrophils and monocytes/macrophages (Bouchon et al., 2000; Bouchon et al., 2001; Colonna, 2003; Colonna & Facchetti, 2003; Wong-Baeza, 2006; Yasuda et al., 2008). A noteworthy finding in these previous studies indicates monocyte expression of TREM-1 is increased during inflammation (Bostanci et al., 2011; Cavaillon, 2009; Ferat-Osorio et al., 2009; Wong-Baeza, 2006). TREM-1 is thought to be a key regulator of the amplitude, rather than the initiator, of the immune response to bacterial challenge. Despite several investigations, its natural ligands remained elusive for many years (Syed et al., 2010). Toll-like receptor ligands were suspected to induce the expression of TREM-1 but recently Read et al (2015) identified the neutrophil peptidoglycan recognition protein 1(PGLYRP1) as a functional ligand for TREM-1. Membrane bound TREM-1 is highly up-regulated in bacterial, fungal, viral, and parasitic infections (de Oliveira Matos et al., 2020; Syed et al., 2010; Yasuda et al., 2008), and as such, it has been used as a marker of infection in adults and children (Garofoli et al., 2010).

Interestingly, ongoing research has also shown that during inflammation mTREM-1 expression is not limited to myeloid cells. It is also expressed on the surface of dendritic cells, vascular smooth cells, and some keratinocytes (Bisson et al., 2012; Rai et al., 2016; Rao et al., 2016). TREM-1 has also been detected in bronchial, corneal, gastric epithelial cells, and hepatic endothelial cells (Barrow et al., 2004; Chen et al., 2008; Rigo et al., 2012; Schmausser et al., 2008). In addition, it has been detected also in gingiva epithelial cells (Chen et al., 2017). Other studies have shown that TREM-1 is enhanced/elevated in atherosclerosis (Joffre et al., 2016; Zysset et al., 2016), myocardial infarction (Boufenzer et al., 2015), IBD/colitis (Schenk et al., 2007), gout (Lee et al., 2016), rheumatoid arthritis (Murakami et al., 2009), renal fibrosis (Lo et al., 2014), pancreatitis (Ferat-Osorio et al., 2009; Yasuda et al., 2008) and psoriasis(Hyder et al., 2013).
3.4 Soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1)
Apart from the membrane bound form (30 kDa), a soluble TREM-1 variant (sTREM-1) (27 kDa) has been detected in mouse and human serum (Gibot et al., 2005; Gibot, Kolopp-Sarda, Béné, Bollaert, et al., 2004). The origin of sTREM-1 has been explained with two hypotheses (Tammaro et al., 2017). Upon transcription, alternative splicing of the Trem1 gene results in the synthesis of a smaller (27 kDa) soluble protein, sTREM-1, which contains only the immunoglobulin-like domain (Ig-like domain), seen on the left side of Figure 11 (Palazzo, Simpson, & Schnapp, 2012; Tammaro et al., 2017). The canonical translation process produces the TREM-1 receptor protein, which consists of the Ig-like domain and a transmembrane (TM) domain. The proteolytic cleavage of this receptor by the MMPs, results in the generation of sTREM-1 protein, shown on the right side of Figure 11 (Gómez-Piña et al., 2007; Tammaro et al., 2017). The MMPs are responsible for the proteolytic cleavage of the membrane bound TREM-1 to soluble TREM-1 (Nylund et al., 2018; Räisänen et al., 2020). The activated matrix metalloproteinase-8 (aMMP-8) is known to be the most responsible MMP that processes membrane bound TREM-1 to sTREM-1 detected and reflected in biologic body fluids such as serum and saliva (Räisänen et al., 2020). Also, MMP-9 cleaves TREM-1 to sTREM-1 (Weiss et al., 2017).

Figure 12: TREM-1 receptor and soluble protein regulation. (Adapted from Tammaro et al (2017)).

sTREM-1 has been suggested to be specific to infectious disorders as it is usually secreted in response to infection and can be measured in body fluids (Mahdy et al., 2006), although its presence has been observed in non-infectious diseases (Tammaro et al., 2017). The secretion or shedding of TREM-1 into body fluids was proven by Syed et al (2010) and

So far sTREM-1 has been detected in patient serum and broncho alveolar lavage (BAL) fluid and has been used as a diagnostic marker of infectious inflammatory conditions, especially during severe sepsis and pneumonia (Gibot & Cravoisy, 2004b; Gibot, Kolopp-Sarda, Béné, Cravoisy, et al., 2004). Other studies have indicated that the expression of sTREM-1 is upregulated in saliva and gingival crevicular fluid (GCF) of patients with periodontitis (Belibasakis et al., 2014; Bisson et al., 2012; Bostanci, Oztürk, et al., 2013; Räisänen et al., 2020; Willi et al., 2014). Nylund et al (2018) demonstrated that sTREM-1 was significantly upregulated in patients with oral inflammation and kidney disease.

Several other studies have shown that sTREM-1 can be measured directly in different other human body fluids such as pleural effusion, sputum, and urine during bacterial infections (Lemarié et al., 2015; Rohde et al., 2012; Su et al., 2015).

Even though many studies showed that body fluids from patients with non-infectious disorders showed no increase in sTREM-1 levels there is one conflicting study that detected elevated sTREM-1 levels in the GCF of both elderly patients with gingivitis or periodontitis and healthy participants (Öztürk et al., 2016).

The biological role function of sTREM-1 is still elusive. Some research studies have suggested that it acts as an anti-inflammatory decoy receptor that binds potential mTREM-1 ligands (Baruah et al., 2015; Roe et al., 2014).

The diagnosis of CU is difficult and complicated. Current methods are clinically, and laboratory based, and these have been discussed in detail in Chapters 1 and 2.

In recent years numerous authors have noted the essential role of sTREM-1 in bacterial, viral, parasitic, and fungal infections (de Oliveira Matos et al., 2020). They have strongly suggested that it can be used as a diagnostic or prognostic marker of infection in adults and children (Garofoli et al., 2010; Lemarié & Gibot, 2020). As far as it is known, there are currently no studies investigating sTREM-1 concentrations in the saliva of chronic urticaria patients. Oral infection is one of the triggers suspected to flare up CU.

Investigating sTREM-1 as a biomarker could help identify cases of chronic urticaria which may be associated with infection even though other studies have shown increased TREM-1 in sterile inflammation, allergic asthma (Bucova et al., 2012).
This study aims to determine and compare the levels of sTREM-1, IL-8, and IL-3 in the saliva of healthy subjects controls, and patients with chronic urticaria. This study tests the hypothesis that there are significantly higher salivary sTREM-1, IL-8, and IL-3 levels in chronic urticaria patients compared to normal control subjects and that IL-8 levels are correlated with salivary sTREM-1.

3.5 Method and Materials
The ELISA DuoSet Kit by R&D Systems was the immunoassay used for the saliva sample validation. The spike/recovery, linearity experiments and testing of patients and health controls samples as well as reasons for choosing the ELISA test have been described in detail in Chapter 2.

3.6.0 Results

3.6.1 sTREM-1 Spike/Recovery, Linearity Results of Controls and Healthy donors
A solution of high recombinant human TREM-1 concentration (6000 pg/ml) was prepared and further serially diluted to prepare the standard curve. The optical density of the solution was measured and presented as the mean value of two readings (Table 6). A standard curve was generated for the TREM-1 ELISA test using log-log plot curve fitting (Figure 12). A representative graph of a typical TREM-1 standard curve is shown (Figure 12). The top point of the standard curve was still 6000 pg/ml. Over the course of the project, two different lot numbers with different stock solution concentrations (180,000 pg/ml and 140,000 pg/ml) were used. The first set of experiments was done using 180,000 pg/ml standard stock solution concentration. When this stock solution ran out, the second batch of reagents with 140,000 pg/ml standard stock solution concentration was acquired and was used for the second set of experiments. All patient and control samples were processed using a similar standard curve.
A seven-point standard curve using 2-fold serial dilutions in reagent diluent with a high standard of 6000pg/ml was recommended and used in this protocol Table 6.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (pg/ml)</th>
<th>Optical Density (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>6000</td>
<td>2.510</td>
</tr>
<tr>
<td>Standard 2</td>
<td>3000</td>
<td>2.245</td>
</tr>
<tr>
<td>Standard 3</td>
<td>1500</td>
<td>1.643</td>
</tr>
<tr>
<td>Standard 4</td>
<td>750</td>
<td>1.052</td>
</tr>
<tr>
<td>Standard 5</td>
<td>375</td>
<td>0.649</td>
</tr>
<tr>
<td>Standard 6</td>
<td>188</td>
<td>0.421</td>
</tr>
<tr>
<td>Standard 7</td>
<td>93.8</td>
<td>0.284</td>
</tr>
</tbody>
</table>

Table 6: TREM-1 standards results of seven points using 2-fold serial dilutions Data used for the construction of the graph above (Figure 13).

3.6.2 sTREM-1 Spike/recovery Results of Control and Donor Samples Determined by ELISA Test.

Five separate spike/recovery and linearity experiments were repeated over days. The spike/recovery data for control and donor samples in terms of salivary sTREM-1 analysis is shown in Table 7 and Table 8 as percentage recovery. In this case, successful recovery is reached when the mean percentage recovery is between 80 to 120 % based on published data (Tiwari & Tiwari, 2010). This study shows that the percent recovery observed for 3,600 pg/ml spiked control was $113.5 \pm 2.1\%$ on average (range 112-115%) whilst the
percent recovery observed for 3,600 pg/ml spiked healthy donor sample was 88.5 ± 0.7% on average (range 88-89%) (Table 7). The percent recovery observed for 2800 pg/ml spiked control was 96.3 ± 5.1 % on average (range 92-102%) whilst the percent recovery observed for 2,800 pg/ml spiked healthy donor sample was 82.5 ± 2.1% on average (range 81-84%) (Table 8).

<table>
<thead>
<tr>
<th>ELISA RUN</th>
<th>SAMPLE</th>
<th>Protease Inhibitor used</th>
<th>% Recovery (3,600 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 1</td>
<td>N</td>
<td>115</td>
</tr>
<tr>
<td>2</td>
<td>Control 2</td>
<td>N</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td>113.5 ± 1.5</td>
</tr>
<tr>
<td>1</td>
<td>Donor 1</td>
<td>Y</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>Donor 2</td>
<td>Y</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Mean ±SEM</td>
<td></td>
<td>88.5 ± 0.5</td>
</tr>
</tbody>
</table>

Table 7: Spike and recovery results of control and healthy donor sample at a final concentration of 3600 pg/ml as determined by the ELISA test.

<table>
<thead>
<tr>
<th>ELISA RUN</th>
<th>SAMPLE</th>
<th>Protease Inhibitor used</th>
<th>% Recovery (2,800 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Control 3</td>
<td>N</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>Control 4</td>
<td>N</td>
<td>102</td>
</tr>
<tr>
<td>5</td>
<td>Control 5</td>
<td>N</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td>96.3 ± 3.0</td>
</tr>
<tr>
<td>3</td>
<td>Donor 3</td>
<td>Y</td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>Donor 4</td>
<td>y</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td>82.5 ± 1.5</td>
</tr>
</tbody>
</table>

Table 8: Spike and recovery results of control and healthy donor samples at a final concentration of 2,800 pg/ml as determined by the ELISA test.

3.6.3 sTREM-1 Linearity Results of Control and Donor Samples Determined by ELISA Test.

Good linearity in the concentration measured was observed at all dilutions for spike controls using 3,600pg/ml except for the 1:8 dilution sample of control 2. The mean percent recovery for the 1,800 pg/ml (1:2) spiked control sample was 108 ± 5.7 % (range 104-112%) whilst for the 900 pg/ml (1:4) spiked control sample was 104 ± 2.8 % (range
The mean percent recovery for the 450 pg (1:8) spiked control sample was 83 ± 34% (range 59-107) (Table 9).

Good linearity can be seen at all dilutions for all spiked donor samples except for the 1:4 dilution of donor 1. The mean percent recovery at 1,800 pg/ml (1:2) spiked donor sample was 93 ± 11.3% (range 85-101%) whilst for the 900 pg/ml (1:4) spiked donor sample it was 112 ± 24% (range 95-129%) (Table 9). The mean percent recovery for the 450 pg (1:8) spiked donor sample was 103 ± 17% (range 86-120%) (Table 9).

<table>
<thead>
<tr>
<th>ELISA Run</th>
<th>Sample</th>
<th>PI Used</th>
<th>% Recovery 1:0 3600pg/ml</th>
<th>%Recovery 1:2 1800pg/ml</th>
<th>%Recovery 1:4 900pg/ml</th>
<th>%Recovery 1:8 450pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 1</td>
<td>N</td>
<td>112</td>
<td>104</td>
<td>106</td>
<td>107</td>
</tr>
<tr>
<td>2</td>
<td>Control 2</td>
<td>N</td>
<td>115</td>
<td>112</td>
<td>102</td>
<td>59</td>
</tr>
<tr>
<td><strong>Mean±SEM</strong></td>
<td></td>
<td></td>
<td><strong>113.5 ± 1.5</strong></td>
<td><strong>108 ± 4</strong></td>
<td><strong>104 ± 2</strong></td>
<td><strong>83 ± 24</strong></td>
</tr>
<tr>
<td>1</td>
<td>Donor 1</td>
<td>Y</td>
<td>88</td>
<td>85</td>
<td>129</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>Donor 2</td>
<td>Y</td>
<td>89</td>
<td>101</td>
<td>95</td>
<td>86</td>
</tr>
<tr>
<td><strong>Mean±SEM</strong></td>
<td></td>
<td></td>
<td><strong>88.5 ± 0.5</strong></td>
<td><strong>93 ± 8</strong></td>
<td><strong>112 ± 17</strong></td>
<td><strong>103 ± 17</strong></td>
</tr>
</tbody>
</table>

Table 9: Linearity results of spike control and healthy donor samples using the 3,600 pg/ml concentration determined by the ELISA test.
PI=protease inhibitors.

Good linearity was observed at all dilutions for spike control samples using 2800 pg/ml. The mean percent recovery at 1400 pg/ml (1:2) spiked control was 99.3 ± 8.7% (range 92-109%) whilst for the 700 pg/ml (1:4) spiked control it was 102 ± 4.6% (range 98-107%) (Table 10). The mean percent recovery at 350 pg (1:8) spiked control was 105 ± 5.7% (range 99-110) (Table 10). Good linearity was seen at all dilutions for all spiked healthy donor samples except for the 1:8 dilution of donor 2. The mean percent recovery at 1400 pg/ml (1:2) spiked donor sample was 90.5 ± 7.8% (range 85-96%) whilst for the 700 pg/ml (1:4) spiked donor sample it was 87.5 ± 6.4% (range 83-92%) (Table 10). The mean percent recovery for the 350 pg (1:8) spiked donor sample was 54 ± 37% (range 17-91%) (Table 10).
<table>
<thead>
<tr>
<th>ELISA Run</th>
<th>Sample</th>
<th>PI Used</th>
<th>% Recovery 1:0 2,800 pg/ml</th>
<th>% Recovery 1:2 1,400 pg/ml</th>
<th>% Recovery 1:4 700 pg/ml</th>
<th>% Recovery 1:8 350 pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 3</td>
<td>N</td>
<td>95</td>
<td>97</td>
<td>101</td>
<td>107</td>
</tr>
<tr>
<td>2</td>
<td>Control 4</td>
<td>N</td>
<td>102</td>
<td>109</td>
<td>107</td>
<td>110</td>
</tr>
<tr>
<td>3</td>
<td>Control 5</td>
<td>N</td>
<td>92</td>
<td>92</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td>96.3 ± 3.0</td>
<td>99.3 ± 5.0</td>
<td>102 ± 2.64</td>
<td>105 ± 3.3</td>
</tr>
</tbody>
</table>

| 1         | Donor 3 | Y       | 81                        | 85                        | 92                        | 91                        |
| 2         | Donor 4 | Y       | 84                        | 96                        | 83                        | 17                        |
|           | Mean ± SEM |       | 82.5 ± 1.5               | 90.5 ± 5.5               | 87.5 ± 4.5               | 54 ± 37                   |

Table 10: Linearity results of spike control samples and healthy donor samples using 2,800pg/ml as determined by the ELISA test.

Overall, linearity appears to be lost as the samples were diluted down to 1:8 after spiking donor samples with either 3,600pg/ml or 2,800pg/ml. This loss of linearity was observed only at 2 different levels and could be associated with a technical error. In the event of small samples or high values, a decision was made to dilute the test samples 1:2 to obtain valid results.

### 3.6.4 The Accuracy and Precision of the rh-sTREM-1 Determination

Accuracy and Precision experiments were done to check the validity of the ELISA test.

Accuracy is the measure of how close the experimental value is to the true value. Accuracy calculates the deviation of the measured results against the true value. Precision is the measure of how close data values are to each other for several measurements under the same analytical conditions (Andreasson et al., 2015). Precision assesses repeatability & calculates the relative standard deviation. For accuracy and precision, different rh-sTREM-1 concentrations were added to the reagent diluent and healthy donor samples and concentrations were measured.

Determination of sTREM-1 levels using the ELISA test for the control sample (reagent diluent) and a healthy donor sample were performed multiple times to measure the accuracy and the precision of the test Table 11 and 12.
The results show sTREM-1 mean concentrations of 2698 ±150 pg/ml at 2800 pg/ml, 1381 ±157 at 1400 pg/ml, 707± 54 at 700 pg/ml, and 364 ±27 pg/ml at 350 pg/ml of the control sample (reagent diluent) Table 11. The results show sTREM-1 mean concentrations of 2313±47 pg/ml at 2800 pg/ml, 1266 ±106 pg/ml at 1400 pg/ml, 612± 47 pg/ml at 700 pg/ml and 191 ±183 pg/ml at 150 pg/ml of the healthy donors Table 12. 6000 pg/ml rh-sTREM-1 was employed and further two-fold serially diluted.

The accuracy of the investigated sTREM-1 results was determined by standard addition comprising serial dilutions of the reference standard (recombinant human sTREM-1) in reagent diluent and healthy donor saliva. The overall recovery expected was 100% if the addition of diluent and saliva did not influence sTREM-1 detection, indicating accurate results in terms of absolute quantity and exclusion of matrix effects.

The percent recovery showed good accuracy of 96.3± 3.0 % at 2800 pg/ml, 99.3± 5.0% at 1400 pg/ml, 102± 2.64% at 700 pg/ml and 105± 3.3% at 350 pg/ml in the reagent diluent Table 11. The percent recovery showed good accuracy of 82.5± 1.5% at 2800 pg/ml, 90.5± 5.5% at 700 pg/ml, 87.5± 4.5 with exception of a low accuracy of 54± 37% at 350 pg/ml in the healthy donors Table 12. The precision of rh-sTREM-1 determination was carried out by mean CV calculations. The precision of the investigated sTREM-1 test was also determined by standard addition comprising serial dilutions of the reference standard (recombinant human sTREM-1) in reagent diluent. No precision was performed on donor saliva samples because they were from different donors. Precision must be estimated from the same experiment in combination with replicate values (several measurements of the same sample under the same conditions) of several independent experiments following identical procedures (Hasibeder et al., 2015). sTREM-1 concentrations showed overall high precision, not exceeding a coefficient of variation (CV) of 10%, except for the dilution of 1:2 which has a CV of 11.4%) (Table 11).

<table>
<thead>
<tr>
<th>Control</th>
<th>2800 (1:0)</th>
<th>1400 (1:2)</th>
<th>700 (1:4)</th>
<th>350 (1:8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2647</td>
<td>1286</td>
<td>667</td>
<td>353</td>
</tr>
<tr>
<td>4</td>
<td>2867</td>
<td>1563</td>
<td>768</td>
<td>395</td>
</tr>
<tr>
<td>5</td>
<td>2580</td>
<td>1294</td>
<td>686</td>
<td>346</td>
</tr>
<tr>
<td>Mean +3SD</td>
<td>2698 ±150</td>
<td>1381 ±157</td>
<td>707± 54</td>
<td>364 ±27</td>
</tr>
<tr>
<td>CV</td>
<td>5.6%</td>
<td>11.4%</td>
<td>7.6%</td>
<td>7.4%</td>
</tr>
<tr>
<td>Accuracy % Recovery</td>
<td>96.3± 3.0</td>
<td>99.3± 5.0</td>
<td>102± 2.64</td>
<td>105± 3.3</td>
</tr>
</tbody>
</table>

Table 11: The precision and accuracy of control samples as determined using the ELISA.
<table>
<thead>
<tr>
<th>Donor</th>
<th>2800 (1:0)</th>
<th>1400 (1:2)</th>
<th>700 (1:4)</th>
<th>350 (1:8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2280</td>
<td>1191</td>
<td>645</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>2346</td>
<td>1341</td>
<td>578</td>
<td>61</td>
</tr>
<tr>
<td>Mean +3SD</td>
<td>2313±47</td>
<td>1266 ±106</td>
<td>612± 47</td>
<td>191 ±183</td>
</tr>
<tr>
<td>Accuracy % Recovery</td>
<td>82.5± 1.5</td>
<td>90.5± 5.5</td>
<td>87.5± 4.5</td>
<td>54± 37</td>
</tr>
</tbody>
</table>

Table 12: The accuracy for healthy donor saliva samples as determined using the ELISA test.

### 3.6.5 Patients' UAS7 Score Results

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>43</td>
<td>34</td>
</tr>
<tr>
<td>UAS7 Mean +SD N=16</td>
<td>12.72+9.03</td>
<td>N/A</td>
</tr>
<tr>
<td>No (%) of participants with UAS7 &gt;6</td>
<td>12(28)</td>
<td>N/A</td>
</tr>
<tr>
<td>No (%) of participants with UAS7 &lt;6</td>
<td>4(9)</td>
<td>N/A</td>
</tr>
<tr>
<td>No (%) of participants with UAS7=0</td>
<td>5(12)</td>
<td>N/A</td>
</tr>
<tr>
<td>No (%) of participants without UAS7</td>
<td>22(51)</td>
<td>34</td>
</tr>
</tbody>
</table>

Table 13: UAS7 score obtained from patients’ notes.

Out of forty-three patients, twenty one patients, representing 49%, had UAS7 scores whilst twenty two (51%) patients did not have a UAS7 score (Table 13). Those who did not have a UAS7 score were either naïve patients or did not complete the UAS7 score sheets. All patients with UAS7 scores were on omalizumab and presented as follows: Five patients (12%) had a UAS7 score of zero, indicating that they did not present with neither wheals nor itching; Four patients (9%) had a UAS7 less than 6, indicating good control of the disease; Sixteen patients had a low UAS7 mean score of 12.72+9.03 indicating a moderate control of the disease. Overall, there was great variation in terms of UAS7 scores in this group of patients with some having high scores compared to others. However, regardless of the lower UAS7 score, a variety of low and high results was observed in patients on omalizumab treatment N=27 and naïve patients N=15.

### 3.6.6 Analysis of CRP, ESR, C3, C4, and Vitamin D Tests in Blood

Routine blood tests were performed to measure ESR, CRP, FBC, C3C4, and Vitamin D in CU patients.
Routine blood tests were performed on CU patients to determine the levels of ESR, CRP, FBC, C3C4, and vitamin D. The results show that all tests were normal except for Vit D3 which was low (Table 14). Samples from healthy controls were not tested. The reference ranges used in Table 14 are used in many laboratories including the UHS Pathology Lab, with the Vitamin D results interpreted using the ranges shown below.

* <30nmol/L: Consistent with deficiency

30-50nmol/L: May indicate deficiency

>50nmol/L: Adequate level

>374nmol/L: Toxicity possible

3.6.7 Correlations
Correlations were determined between salivary sTREM-1 and other biomarkers. The results show that there were no significant correlations between salivary sTREM-1 and plasma biomarkers (ESR, CRP, C3, C4, VITD) in the patient group using non-parametric Spearman rank correlation testing.

3.7 Determination of sTREM-1 Levels in Saliva samples.
Analysis of sTREM-1 in the samples from healthy subjects found that 59% of the samples had levels below LOD of 23.2 pg/ml. However, a smaller percentage (20%) were below the LOD in the untreated CU group Table 15. The number of values (n) below the LOD are indicated as a ratio of the number (N) of samples analysed and percentage (%) of samples in Table 15.

<table>
<thead>
<tr>
<th>sTREM-1 (LOD=23.2 pg/ml)</th>
<th>Healthy controls</th>
<th>All CU</th>
<th>CU untreated</th>
<th>CU OMA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;LOD (n/N)</td>
<td>20/34</td>
<td>19/43</td>
<td>3/15</td>
<td>15/27</td>
</tr>
<tr>
<td>&lt;LOD (%)</td>
<td>59</td>
<td>44</td>
<td>20</td>
<td>56</td>
</tr>
</tbody>
</table>

Table 15: Ratio and percentages < LOD in healthy controls, all CU, CU untreated and CU OMA treated.
3.7.1 Fisher Exact Test sTREM-1 Data Analysis

In the first statistical analysis of the data, the Fisher Exact Test was applied to compare the proportions of samples from healthy controls and CU patients (untreated and treated with Omalizumab) with detectable or undetectable levels of sTREM-1.

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>negative TREM-1</th>
<th>positive TREM-1</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Controls</td>
<td>20</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>CU</td>
<td>19</td>
<td>24</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>38</td>
<td>77</td>
</tr>
</tbody>
</table>

Table 16: sTREM-1 contingency table for all samples, healthy controls, and CU

<table>
<thead>
<tr>
<th>Test</th>
<th>Fisher's exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.2535</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant (P &lt; 0.05)?</td>
<td>No</td>
</tr>
<tr>
<td>Effect size</td>
<td>Value</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.5128</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.6316</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.5882</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.5581</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>1.392</td>
</tr>
</tbody>
</table>

Table 17: Fisher’s exact test p-value and statistical significance

The Fisher Exact Test was also applied to compare the proportions of samples from healthy controls and untreated CU patients with detectable or undetectable levels of sTREM-1

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>sTREM-1 negative</th>
<th>sTREM-1 positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>20</td>
<td>14</td>
<td>34</td>
</tr>
<tr>
<td>CU untreated</td>
<td>3</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>26</td>
<td>49</td>
</tr>
</tbody>
</table>

Table 18: sTREM-1 contingency table for all healthy samples and untreated CU
Table 19: Fisher’s exact test $p$-value and statistical significance comparing healthy controls with untreated CU patients

The Fisher Exact Test was also applied to compare the proportions of samples from healthy controls and OMA-treated CU patients with detectable or undetectable levels of sTREM-1

Table 20: sTREM-1 contingency table for all healthy samples and OMA-treated CU

Table 21: Fisher’s exact test $p$-value and statistical significance comparing healthy controls with OMA-treated CU patients
The Fisher Exact Test was also applied to compare the proportions of samples from untreated and OMA-treated CU patients with detectable or undetectable levels of sTREM-1.

Table 22: sTREM-1 contingency table for untreated and OMA-treated CU

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>TREM-1 negative</th>
<th>TREM-1 positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU untreated</td>
<td>3</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>CU OMA treated</td>
<td>15</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>24</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 23: Fisher’s exact test p-value and statistical significance for untreated and OMA-treated CU patients.

<table>
<thead>
<tr>
<th>Test</th>
<th>Fisher’s exact test</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.0493</td>
<td></td>
</tr>
<tr>
<td>P value summary</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
<td></td>
</tr>
<tr>
<td>Statistically significant (P &lt; 0.05)?</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Effect size</td>
<td>Value</td>
<td>95% CI</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.1667</td>
<td>0.05837 to 0.3922</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.5000</td>
<td>0.3143 to 0.6857</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.2000</td>
<td>0.07048 to 0.4519</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.4444</td>
<td>0.2759 to 0.6269</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>0.3333</td>
<td></td>
</tr>
</tbody>
</table>

The Fisher Exact Test indicates a significantly higher proportion of sTREM-1 positive samples in patients with untreated CU compared to healthy controls (p<0.0152), Table 19 and between untreated and OMA-treated patients with CU (p<0.049), Table 23. The Fisher Exact Test indicates no significant higher proportion of sTREM-1 positive samples in all CU patients compared to healthy controls (p>0.2535) Table 17. OMA-treated CU patients have sTREM-1 not significantly (p>0.999) higher than healthy controls values, Table 21.

**3.7.2 Censored sTREM-1 Data Analysis**

The Mann-Whitney test comparing salivary sTREM-1 in all healthy control samples with all CU samples (OMA-treated or untreated) showed a significant difference (p=0.0277)
between groups, Table 24, and values for sTREM-1 were significantly higher in the CU group.

<table>
<thead>
<tr>
<th>P value</th>
<th>0.0277</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exact or approximate P value?</td>
<td>Exact</td>
</tr>
<tr>
<td>P value summary</td>
<td>*</td>
</tr>
<tr>
<td>Significantly different (P &lt; 0.05)?</td>
<td>Yes</td>
</tr>
<tr>
<td>One- or two-tailed P value?</td>
<td>Two-tailed</td>
</tr>
<tr>
<td>Sum of ranks in column A, B</td>
<td>1126, 1877</td>
</tr>
<tr>
<td>Mann-Whitney U</td>
<td>531</td>
</tr>
</tbody>
</table>

Table 24: Mann Whitney test comparing salivary sTREM-1 in all healthy control samples with all CU samples (OMA-treated or untreated)

Figure 14: Salivary sTREM-1 (censored data) comparing control healthy samples with all CU samples.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Urticaria TREM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>34</td>
<td>43</td>
</tr>
<tr>
<td>Minimum</td>
<td>16.41</td>
<td>16.41</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>16.41</td>
<td>16.41</td>
</tr>
<tr>
<td>Median</td>
<td>16.41</td>
<td>32.00</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>37.28</td>
<td>185.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>160.0</td>
<td>1377</td>
</tr>
</tbody>
</table>

Table 25: Values for salivary sTREM-1 (pg/ml) (censored data) in healthy controls and all CU patients
### 3.7.3 ROC Curve Analysis

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>0.6368</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.06263</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.5140 to 0.7596</td>
</tr>
<tr>
<td>P value</td>
<td>0.0402</td>
</tr>
</tbody>
</table>

Table 26: Area under the ROC curve for sTREM-1

![ROC Curve](image)

Figure 15: ROC curve of censored sTREM-1 data for all healthy control and all CU samples (OMA-treated and untreated).

ROC analysis was significant ($p=0.0402$) and the area under the curve was 0.6368, Table 26. This suggests a 64% chance that TREM-1 will correctly distinguish CU patients from normal.

Data from CU patient sample analysis was subsequently grouped according to Omalizumab treatment or not. Kruskal-Wallis ANOVA for the difference between concentrations of sTREM-1 in healthy control saliva samples and those in untreated CU or OMA-treated CU showed a significant ($p=0.0120$) difference between the groups.
Figure 16: Censored sTREM-1 data by treatment group as individual points

<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>CU untreated</th>
<th>CU OMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>34</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Minimum</td>
<td>16.41</td>
<td>16.41</td>
<td>16.41</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>16.41</td>
<td>27.20</td>
<td>16.41</td>
</tr>
<tr>
<td>Median</td>
<td>16.41</td>
<td>89.50</td>
<td>16.41</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>37.28</td>
<td>237.6</td>
<td>176.4</td>
</tr>
<tr>
<td>Maximum</td>
<td>160.0</td>
<td>986.9</td>
<td>1377</td>
</tr>
</tbody>
</table>

Table 27: Values for salivary sTREM-1 (pg/ml) (censored data) in healthy controls, CU untreated and all OMA- CU treated patients

<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>CU untreated</th>
<th>CU OMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>34</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Minimum</td>
<td>16.41</td>
<td>16.41</td>
<td>16.41</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>16.41</td>
<td>27.20</td>
<td>16.41</td>
</tr>
<tr>
<td>Median</td>
<td>16.41</td>
<td>89.50</td>
<td>16.41</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>37.28</td>
<td>237.6</td>
<td>176.4</td>
</tr>
<tr>
<td>Maximum</td>
<td>160.0</td>
<td>986.9</td>
<td>1377</td>
</tr>
</tbody>
</table>

Table 28: Kruskal-Wallis test ANOVA for the difference between concentrations of sTREM-1 in healthy control saliva samples and those in untreated CU or OMA-treated CU
Individual values are shown in Figure 16. There is considerable overlap. Dunn's multiple comparisons test showed that sTREM-1 values (median (interquartile range)) for untreated CU (89.5 (27.2-237.6) pg/ml), but not samples from OMA-treated CU patients (16.41 (16.41-176.4) pg/ml), were significantly higher than healthy control values (16.41 (16.41-37.28) pg/ml). There was no significant difference between controls and OMA-treated patients, but since these are not paired samples, an effect of therapy is not proved.

Additionally, ROC analysis for salivary sTREM-1 in untreated CU compared to healthy controls was highly significant ($p=0.0034$) and the area under the curve was 0.7647 Table 29. This suggests a 76% chance that TREM-1 will correctly distinguish CU patients from normal healthy controls.

![ROC analysis for sTREM-1 in saliva samples from healthy controls vs untreated CU patients.](image)

Table 29: Area under the ROC curve for sTREM-1 in saliva samples from healthy controls vs untreated CU patients.

<table>
<thead>
<tr>
<th>Area</th>
<th>0.7647</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. Error</td>
<td>0.07825</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.6113 to 0.9181</td>
</tr>
<tr>
<td>$P$-value</td>
<td>0.0034</td>
</tr>
</tbody>
</table>
3.7.4 Sensitivity, Specificity, and Likelihood Ratio of Salivary sTREM-1

Table 30: Sensitivity, specificity, and likelihood ratio of salivary sTREM-1 raw data in the naïve patients

A cut off value for sTREM-1 that gave the best combination of sensitivity and specificity was chosen from the raw data, calculated from the GraphPad prism Table 30. A salivary sTREM-1 value of > 27.15 pg/ml gave 80.00% sensitivity (54.81% to 92.95%) CI, and 67.65% specificity (50.84% to 80.87%). The likelihood ratio was 2.473, indicating that the test is more likely to be positive in disease. Higher sensitivity is desirable in diagnostic screening and a higher specificity is desirable in a screening setup.

3.7.5 Negative Predictive Value and Positive Predictive Value of sTREM-1

The negative predictive value (NPV) and the positive predictive value (PPV) were also calculated based on a projected point prevalence in the population for CU of 1% (Powell et al., 2015). The PPV is low, only 2.4% but the NPV is high (99.7%).

3.8.0 Discussion

TREM-1, a transmembrane receptor, is a founding member of the immunoglobulin superfamily mainly expressed on neutrophils and monocytes/macrophages (Bouchon et al., 2000; Bouchon et al., 2001; Colonna, 2003; Colonna & Facchetti, 2003; Gibot & Cravoisy, 2004a). Monocytes and neutrophils are the major cells of the innate immune system that wage a response against bacterial infections. TREM-1 expression is increased in response to the presence of bacteria, viruses, parasites, and fungi (de Oliveira Matos et al., 2020),
and soluble TREM-1 is released, and its concentration increases rapidly in human body fluids (Dimopoulou et al., 2012; Lemarié et al., 2015).

Research studies carried out over the last two decades identified TREM-1 as a potential biomarker for the diagnosis of sepsis or bacterial, viral, parasitic, and fungal infections (Bouchon et al., 2000; de Oliveira Matos et al., 2020; Michel et al., 2017). These studies showed that membrane bound TREM-1 is highly upregulated in bacterial and fungal infections (Bouchon et al., 2001; Colonna & Facchetti, 2003; Gibot & Cravoisy, 2004b) and as such TREM-1 is contemplated to be a key amplifier of the magnitude rather than the initiation of the immune response to bacterial challenge and the consequent production of proinflammatory cytokines (Willi et al., 2014). Furthermore, it has been found that TREM-1 is increased in non-infectious inflammatory processes such as psoriasis and ulcerative colitis (Tammaro et al., 2017).

Apart from the membrane bound form, a soluble TREM-1 variant (sTREM-1) has been detected in mouse and human serum (Gibot et al., 2005; Gibot, Kolopp-Sarda, Béné, Bollaert, et al., 2004). Importantly, elevated levels of sTREM-1 have also been detected in saliva, and gingival crevicular fluid of some patients with oral infection and inflammation (Belibasakis et al., 2014; Bostanci, Oztürk, et al., 2013; Willi et al., 2014). sTREM-1 has now been identified as a novel potential biomarker for the diagnosis of sepsis and/or the development of serious infectious complications (de Oliveira Matos et al., 2020; Lemarié & Gibot, 2020). The origin of sTREM-1 has been explained with two hypotheses (Tammaro et al., 2017). The first hypothesis implicates a splice variant of the TREM-1 mRNA (Palazzo, Simpson, & Schnapp, 2012), whereas the second hypothesis proposes that sTREM-1 is generated through the proteolytic cleavage of the mature cell surface-anchored TREM-1 (Gómez-Piña et al., 2007).

3.8.1 sTREM-1 Key Findings

The present study was carried out to investigate the potential usefulness of salivary sTREM-1 as a biomarker of chronic urticaria. As far as it is known, this is the first study to compare total levels of sTREM-1 in the saliva of healthy subjects and chronic urticaria patients. sTREM-1 was detected at a low concentration in the saliva of healthy controls. The median sTREM-1 in controls was 16.41 pg/ml (range 16.41 - 37.28 pg/ml). However, and in striking contrast, the concentration of sTREM-1 was significantly ($p$ value= 0.0277) increased in the saliva of patients with chronic urticaria, suggesting the presence of oral
bacterial infection in these patients. The median sTREM-1 in patients with CU was 32.0 pg/ml (range 16.41-185.7 pg/ml). The ROC curve for salivary TREM-1 was significant, (p value =0.0402), the area under the ROC curve 0.6368, std. error 0.06263, 95% confidence interval 0.5140 to 0.7596. This suggests a 64% chance that TREM-1 will correctly distinguish CU patients with oral bacterial infections from normal.

The patients were further subdivided into naïve (untreated patients), omalizumab treated, and healthy control groups. sTREM-1 was significantly (p=0.0120) higher in naive patients than healthy controls. Patients treated with omalizumab have sTREM-1 not significantly higher than healthy control values. The ROC curve for sTREM-1 in untreated patients is significant (p=0.0046), the area under the ROC curve 0.7647, std. error 0.07825, 95% confidence interval 0.6113 to 0.9181. This suggests a 76% chance that TREM-1 will correctly distinguish CU patients with oral infections from normal, an increase of 13% from when all samples were analysed. A salivary sTREM-1 value of > 27.15pg/ml gave 80.00% sensitivity (54.81% to 92.95%) CI, and 67.65% specificity (50.84% to 80.87%). The likelihood ratio was 2.473, indicating that the test is more likely to be positive in disease. This higher sensitivity strengthens the diagnostic potential of sTREM-1in CU patients with suspected oral bacterial infections.

The ROC analysis indicates salivary sTREM-1 could be a diagnostic marker for CU with suspected oral infections, but the positive predictive value (PPV) will be low and NPV will be high for CU which has a low prevalence. The PPV and the NPV are influenced by the prevalence of a disease in a population that is being tested. In a high prevalence setting, it is more likely that the persons who test positive truly have the disease than if the test is performed in a population with low prevalence.

The British Society for Allergy and Clinical Immunology guidelines for the management of chronic urticaria and angioedema (BSACI guidelines) state that the lifetime prevalence of chronic urticaria is 0.5–1% (Powell et al., 2015). Although rarely life-threatening, CU significantly reduces the quality of life. The NPV and the PPV were calculated based on a projected point prevalence in the population for CU of 1%. The PPV is low, only 2.4% but the (NPV) is high (99.7%). A low sTREM-1 value would rule out CU with suspected oral infection, and the value of the test is largely in the NPV. CU diagnosis therefore could not be based on a single salivary sTREM-1 but would need confirmatory tests.
The sTREM-1 concentration (median (interquartile range)) in saliva from patients with untreated CU was (89.5 (27.2-237.6) pg/ml) and from healthy controls was (16.41 (16.41-37.28) pg/ml) in this study. The sTREM-1 median value (16.41 pg/ml) in the control group of this study is lower compared to a previous study (median 184.3 pg/ml) done in healthy adolescents (Raivisto et al., 2020). Also, Bostanci et al (2013) study had a higher mean sTREM value in the healthy control group (384.60+-115.81pg/ml). In their study, Bostanci et al (2013) collected saliva samples from 59 subjects (control: n = 18, chronic periodontitis: n = 20, generalized aggressive periodontitis: n = 21). The concentrations of sTREM-1 in these samples were further analysed by ELISA. sTREM-1 was detected in all the samples. The mean concentrations were 384.60 ± 115.81 pg/ml in the control group, 1272.38 ± 138.91 pg/ml in chronic periodontitis (3.3-fold higher than the control) and 2179.95 ± 306.14 pg/ml in generalized aggressive periodontitis (5.6-fold higher than the control). These values were measured in whole saliva samples expectorated into a Falcon tube using an R&D ELISA. It is not clear if the DuoSet ELISA or the Quantikine ELISA was used in their study. It is likely the DuoSet ELISA was used as the LOD was given as 46 pg/ml. The Quantikine ELISA has a lower LOD of 15.2 pg/ml.

Another high mean sTREM value was also recently detected in the healthy children control group (Yucel et al., 2020). In their study, Yucel et al (2020) measured mean salivary concentrations (±SD, pg/mL) of TREM-1, 49.10±47.64 in the cystic fibrosis-periodontally healthy group, 169.44±123.26 in the cystic fibrosis-gingivitis group, 38.04±42.13 in the control-periodontally healthy group, and 242.17±141.21 in the control-gingivitis group (3.4-fold higher in cystic fibrosis-gingivitis compared with cystic fibrosis-periodontally healthy and 6.3-fold higher in control-gingivitis compared with control-periodontally healthy).

Thus, significantly increased levels of sTREM-1 were associated with gingivitis, in both healthy and CF patients, but were not associated with CF. Salivary sTREM-1 levels were highly variable in control groups, 38.04±42.13 in healthy control, and 49.10±47.64 in CF without gingivitis. This study found similar variable low concentrations (16.41 pg/ml) in the healthy saliva samples. In Yucel et al (2020) study, TREM-1 was detectable in only 75% of saliva samples, and the study used the same Human TREM-1 DuoSet ELISA, R&D Systems (DY1278B) and a level of detection of 23.1 pg/ml.
Regrettably, it is difficult to compare the control values of this study with Bostanci et al (2013) and Yucel et al (2020) because they used mean values instead of median. A mean value can be increased by one or two exceptionally high values. The sTREM-1 median value (32.0 pg/ml) in the patient group of this study was also lower compared to Raivisto et al (2020) study (median 333.1 pg/ml). Bostanci et al (2013) and Yucel et al 2020 had higher mean values in the patient group, 1272.33+138.91 pg/ml and 242.17+141.21 pg/ml, respectively. TREM-1 in these previous studies was measured in whole saliva expectorated into a tube centrifuged at 10,000 g x 15 min and frozen immediately, with no protease inhibitors added. This study used the salivette collection tube centrifuged at 4 °C, 10,000 g x 15 minutes and frozen immediately, with protease inhibitors added. The salivette collection system may affect recovery of sTREM-1.

### 3.8.2 Potential Impact of sTREM-1 on Clinical Practice

As far as it can be ascertained the association of sTREM-1 with chronic urticaria is not yet known. Considering the increased secretion of salivary sTREM-1 observed in subjects with chronic urticaria disease compared with healthy subjects, the higher levels of sTREM-1 observed in this study are likely to be a consequence of inflammation present in the gums, because of infection, or reflect a general mucosal inflammation, of the chronic urticaria patients. Hence, the sTREM-1 data has the potential to indicate that bacterial infection is a tractable trigger for CU even though no infection analysis was done in the patients in this study. Salivary sTREM-1 levels are known to be higher in bacterial infections than in healthy individuals (Rudick et al., 2017) hence the low sTREM-1 levels observed in healthy controls are thought to be a result of the absence of bacterial infections in the oral tissues. However, it is important to note that inflammation in CU may not be related to bacterial infection. Increased salivary sTREM-1 in CU may reflect autoimmunity, allergy, stress, and other factors that predispose patients with CU to sterile inflammation (Bansal & Bansal, 2019).

Now, very few clinicians take their time to examine the mouths and teeth of patients with chronic urticaria when they come for their clinical appointments. A recent case study showed the resolution of CU following treatment of oral infection (Tadros et al., 2018). Therefore, this study will raise awareness to all clinicians who see chronic urticaria patients in allergy clinics and other primary care clinics to perform oral examinations so that this tractable trigger, if present, for chronic urticaria is not missed.
Previous studies show that the expression of sTREM-1 is upregulated in the saliva of patients with periodontitis without chronic urticaria. In these studies, sTREM-1 levels were found to be higher in subjects with periodontitis than in healthy subjects (Bostanci & Belibasakis, 2012; Bostanci, Oztürk, et al., 2013; Bostanci et al., 2011; Räisänen et al., 2020; Willi et al., 2014). Nylund et al (2018) demonstrated that sTREM-1 was significantly upregulated in patients with oral inflammation and kidney disease. Belibasakis et al (2014) investigated sTREM-1 on gingival crevicular of patients diagnosed with periodontal disease and found these levels to be higher than those of healthy control subjects. Interestingly, elevated sTREM-1 levels have been detected in the GCF of both elderly patients with gingivitis or periodontitis and healthy participants (Öztürk et al., 2016). The elevation in both groups of participants was suggested to be caused by a dysregulated immune response in elderly populations due to the compromised function of monocytes and macrophages.

Bostanci et al (2013) determined the relation between sTREM-1 levels in serum and saliva in patients with periodontal disease and reported that sTREM-1 was raised in both serum and saliva. Also, a review done by Rudick et al (2017) showed that oral infections were associated with systemic inflammation. However, this study did not compare oral and systemic sTREM-1. Even though there is a convincing body of evidence that indicates elevated sTREM-1 is detected in other fluids of patients suffering from bacterial infectious diseases (Gibot et al., 2005; Gibot, Cravoisy, et al., 2004; Kofoed et al., 2007; Liu et al., 2007), some contrasting results have however been obtained with regards to the usefulness of sTREM1 concentration as a biomarker to identify bacterial infections and critically ill patients (Kwofie et al., 2012; Michel et al., 2017)

Increased levels of sTREM-1 in this study were obtained in line with previous studies although it is difficult to make comparisons between this study and previous studies. Patients in the previous studies did not have chronic urticaria. It is noteworthy that even on Omalizumab treatment sTREM-1 was still detectable in some chronic urticaria patients. This could be attributed to an unnoticed ongoing oral infection or inflammation. Analysing soluble TREM-1 in the saliva of chronic urticaria patients is a new procedure. The findings in this study suggest the capacity of sTREM-1 to be helpful as a diagnostic marker in CU patients with suspected oral infection. Several potential advantages of using salivary sTREM-1 as a diagnostic marker are, its cost effectiveness, it is easy, and non-invasive. The ELISA measurement of sTREM-1 is rapid and provides accurate results within hours
of getting a sample from patients. This knowledge heralds new opportunities for the manipulation of sTREM-1 and its development to a new diagnostic biomarker in CU patients. It has also provided a new perspective on the functioning of the immune system in these patients. The new sTREM-1 information and what is yet to be learned about it could have important clinical implications for the diagnosis, monitoring, and treatment of CU patients. The patient and financial benefits of this new procedure could be enormous. The technique used for its determination is rapid, inexpensive, and can be performed for small batches of specimens.

Soluble TREM-1 should not be used as the sole marker of bacterial infection but should be used with a panel of clinical signs and other biomarkers to obtain a greater predictive value of bacterial infection. Besides, soluble TREM-1 does not determine the causative organism and therefore does not preclude the clinician from performing microbiological studies. Setting up and validating the soluble TREM-1 determination method might be the start of a new era of using this method to diagnose bacterial infections in many other conditions that are difficult to diagnose using the routine methods that are currently in use.

Many studies have been evaluating many proteins as biomarkers for bacterial and fungal infections. Some studies have combined the use of biomarkers and fever, appearance of tracheal secretions, bacterial culture, and white cell count to get a comprehensive picture of the patient’s status in pneumonia (Pugin et al., 1991; Singh et al., 2000; Wood et al., 2003). These biomarkers could be used to monitor and treat very sick patients before culture assays can give a definitive diagnosis.

Bacterial culture is the gold standard method for detection and isolation of microorganisms in body fluids and airways of patients suspected of bacterial infections (Baker et al., 1996; Gibot, 2006, 2009; Gibot, Cravoisy, et al., 2004; Loftus et al., 2016; Rello et al., 2014; Wunderink, 1998). Regrettably, there were no culture tests done in this study. Bacterial culture is discussed in detail in chapter 6.

In this study, the levels of the routine blood tests ESR, CRP, C3, C4, and Vitamin D3 were within the normal reference range with vitamin D3 on the lower end. The routine blood tests results are discussed in detail in Chapter 6.

The slow recruitment of naïve patients coupled with research time constraints resulted in the study focusing on medicated CU patients. Most of the patients (n=27) in this study were on Omalizumab treatment and a few were naïve patients (n=15) with no record of
treatment. It was not known for how long these patients had been on omalizumab. Omalizumab is discussed in detail in Chapter 2 and Chapter 6.

It is still very much debatable to use sTREM-1 as a diagnostic marker as well as to prognosticate the outcome of a septic patient by determination of its concentration (Lemarié et al., 2015). Immunoblot techniques were the first methods used to detect sTREM-1 (Gibot, Cravoisy, et al., 2004; Gibot, Kolopp-Sarda, Béné, Bollaert, et al., 2004), however, since 2005 commercially available sandwich ELISAs have been used increasingly (Determann et al., 2005; Giamarellos-Bourboulis et al., 2006; Radsak et al., 2007). There are large variations observed both during the preanalytical and the analytical period which has caused challenges to compare the different techniques used to measure sTREM-1 concentrations. Consequently, some commercial sTREM-1 kits have been withdrawn from the market in the past due to unreliable results (Gibot, 2009; Lemarié et al., 2015). Worryingly huge differences have also been observed in the detected sTREM-1 concentrations under similar pathological conditions (Dopheide et al., 2013).

Hasibeder et al (2015) investigated R&D sTREM-1 ELISAs (Radsak, R&D with/without protein buffer and Hycult Biotech) and found certain characteristics in precision and accuracy. The different sTREM-1 ELISAs showed precision, not exceeding a coefficient of variation (CV) of 20% (Radsak 15%, R&D 13.6%, R&D+ protein buffer 20%, Hycult Biotech 9%). The recovery accuracy revealed large differences between the investigated ELISAs, ranging from 40 to 84% (Radsak 58.5%, R&D 49.3%, R&D+ pcb 40.6%, Hycult Biotech 84.1%).

They observed that the performance of sTREM-1 was extremely dependent on the protein content of the buffer. They obtained better sTREM-1 results when they used a protein-free buffer. The recovery rate (49.3%) of rh-sTREM-1 was high in the protein-free buffer.

Hasibeder et al (2015) recovery accuracy (40.6% and 49.3%) using the sTREM-1 ELISAs was much lower than the recovery accuracy (82.5 ± 1.5%) of this study. The precision in this study (8.0%) was much better compared to Hasibeder et al (2015) study (R&D 13.6%, R&D+ protein buffer 20%). It is difficult to compare these two studies because Hasibeder et al (2015) used serum and this study used saliva for the recovery tests.

As far as it can be ascertained, interfering factors are not yet fully defined for the R&D sTREM-1 ELISA in saliva. sTREM-1 may be degraded by proteases present at sites of inflammation, (Rudick et al (2017)). Protease inhibitors (PIs) were added to all saliva of
participants in this study. The PIs are known to reduce the proteolytic activity of salivary enzymes on sTREM-1. The recovery, linearity, accuracy, and precision of sTREM were within acceptable ranges and hence the ELISA test is suitable for salivary sTREM-1 testing at a 1:2 dilution of saliva. Other endogenous factors are known to be possible interfering factors. These endogenous factors are difficult to detect and eliminate, since they vary from patient to patient, and also from time to time in one patient. They include hyperlipidaemia, nonesterified fatty acids, cross-reacting heterophilic anti-immunoglobulin antibodies, and complement (Weber et al., 1990). Complement components are known to be the most interfering factors as they easily react with several immunoglobulins (Papp et al., 2007). Besides, standardization of sample collection is advisable, because differences in sample logistics have an impact on the outcome of assay results. Repeated freeze/thaw cycles should be avoided because it affects the correct measurement of sTREM-1 (Gibot, 2009). For clinical application and comparative studies, there is a need for further work to be done on salivary sTREM-1 ELISAs. A larger patient sample size is needed to be performed to confirm the results of this study.

3.8.3 Limitations
A limitation of this study is the small number of participants enrolled. The CLSI EP12-A2 ("EP12-A2 User protocol for evaluation of qualitative test performance; approved guideline," 2008) recommends that as a minimum, testing should continue until results from at least 50 positive specimens are obtained and that at least 50 negative specimens using the comparative method should be obtained to determine the specificity of the candidate method. However, although this was the aim of this postgraduate study, it was not possible in the time frame and the study finally recruited 43 patients and 34 controls. Moreover, there were no bacterial cultures done and salivary CRP was not tested. ESR is a test that can only be done using full blood. Future work requires many participants, more salivary tests such as CRP and procalcitonin (PCT). PCT is a protein biomarker for the presence and severity of bacterial infection whose level rises within 12 hours of bacterial involvement and decreases as the host immune system begins to control the infection (Peters et al., 2021). Unlike CRP and ESR PCT levels remain low in non-bacterial causes of infection and inflammation (Wacker et al., 2013). The early diagnosis of bacterial infections remains challenging particularly in patients with a multiplicity of clinical presentations. The specific involvement of soluble TREM-1 solely in cases of bacterial infections would be extremely helpful to discriminate between chronic urticaria patients.
with and without infection. Hence the need to measure sTREM-1 in CU patients with and without known periodontal infection.

3.8.4 Conclusion
A significant increase was observed in the levels of sTREM-1 in the saliva of CU patients relative to healthy control subjects. These elevated levels of sTREM-1 suggest the persistent presence of oral bacterial infection in CU patients. sTREM-1 may play a key role in the host response to infection in the oral cavities of CU patients. Infection in the oral cavity of CU patients is one among many factors that trigger CU hence identification of sTREM-1 may be very important in the prognoses of CU. In contrast, the levels of routine blood tests (ESR, CRP, C3C4, and Vitamin D) were with the normal reference range. Based on the results of this study, there is increasing evidence that salivary sTREM-1 is raised in bacterial infections. It is therefore concluded that salivary sTREM-1 together with other biomarkers will have a progressive important clinical role to identify CU patients with oral bacterial infections. Using it with other biomarkers will help to accelerate diagnosis as well as start a specific therapy and monitor the course of bacterial infections in CU patients.
Chapter 4: Interleukin 8 (IL-8)

Mast cells and basophils are important in the pathology of chronic urticaria. Activated mast cells undergoing degranulation are reported to release some cytokines including IL-8. It is also speculated that oral infection microorganisms and the toxins they produce in periodontal tissue stimulate the formation of IL-8. The potential use of IL-8 as a biomarker of CU necessitated its inclusion in this study.

4.1.0 Cytokines

Interleukins are cytokines that play an important role in communication between inflammatory cells following the activation of the immune system. Cytokines are small soluble proteins that mediate and regulate local, systemic, and inflammatory responses and they participate in many biological events (Ertugrul et al., 2013). They regulate the generation, survival, and function of cells by binding to cell surface receptors (Voehringer, 2012). They are known to engage in the communication between cells of the immune system. They are usually classified according to their functional grouping as: (a) mediators and regulators of innate immunity which act on endothelial cells and leukocytes to stimulate early innate response (b) mediators and regulators of adaptive immunity which act on lymphocytes to stimulate and regulate adaptive responses to specific infections, (c) stimulators of haematopoiesis which act on the bone marrow to stimulate growth and differentiation of leukocytes and lymphocytes. Cytokines are involved in both cellular and antibody-mediated immunity. They direct the growth, development, maturation, activation, and life span of immune cells. They play a major role in the inflammatory and immune responses and several of them display inflammatory and others anti-inflammatory responses.

4.1.1 Chemokines

Chemokines are a group of basic cytokines whose molecules contain cationic amino acids, and their sizes range from 6-14 kDa (Ertugrul et al., 2013; Miller & Mayo, 2017; Singh et al., 2013; Trivedi & Adams, 2018). Under physiological conditions, chemokines can exist as monomers, dimers, or a mixture of monomers and dimers (Das et al., 2010; Nasser et al., 2009). They are expressed and secreted by any stimulated monocytes/macrophages (Kaplan et al., 1987), neutrophils (Gasperini et al., 1999), eosinophils (Dajotoy et al., 2004), B lymphocytes (Kato et al., 2004), including endothelial,
epithelial and stromal cells (Trivedi & Adams, 2018). Bacterial and viral stimuli are known factors involved in the induction of chemokine production. In addition, pro-inflammatory cytokines such as IL-1 and tumor necrosis factor-α also induce chemokines production.

4.1.2 The Structure of Chemokine Classes

![Structure of chemokine classes](https://commons.wikimedia.org/wiki/File:Chemokine.png)

The chemokine molecular structure has four conserved cysteine residues. These cysteine residues are arranged in a pattern that forms two disulphide bonds pairing the first with the third and the second with the fourth cysteines (Zlotnik & Yoshie, 2012). Based on this arrangement, the chemokine superfamily is subdivided into four families grouped according to the pattern of cysteines in the N-terminal region: CXC (alpha), CC (beta), C (gamma), and CX3C(delta) (Ertugrul et al., 2013) Figure 18. Each subfamily comprises different types of chemokines, notably: The C subfamily has 27 types, the CC has 17 types, the CXC has 2 types the CX3C has 1 type. These chemokines subfamilies are distinguished according to the position of the first two cysteines. In the CXC chemokines, one amino acid separates the first two cysteines, whereas in CC chemokines these two
cysteines are adjacent (Baggiolini et al., 1997; Zlotnik & Yoshie, 2012). Chemokine genes are in clusters, CXC chemokines on chromosome 4 and CC chemokines on chromosome 17.

4.1.3 Families of chemokines

Previous studies have revealed a full list of families of both human and mouse chemokines, including their functions, genes, and receptors (Zlotnik & Yoshie, 2012). Different chemokine members activate different white cells through specific chemokine receptors found on these cells, Figure 19. CC activates mononuclear cells, eosinophils, and basophils, CXC activates neutrophils and lymphocytes, C activates T cells and CX3C activates natural killer cells (Ertugrul et al., 2013). Chemokine receptors have been extensively characterised in terms of function and binding properties. These receptors are known as class A G protein chemokine receptors (GPCRs) coupled with the Gαi class of heterotrimeric G proteins, grouped into subfamilies according to the subfamily of their major chemokine ligand (Zlotnik et al., 2006).

Currently, 18 chemokine receptors have been identified that possess the Gαi-dependent chemotactic activity. In addition, 5 nochemotactic receptors have also been described (Zlotnik & Yoshie, 2012). It is important to note that chemokine receptor genes are also located in clusters, with the large cluster being in human chromosome 3. One research study showed that some chemokines ligands tend to be shared by multiple receptors (Nomiyama et al., 2011).
Figure 19: Main chemokine receptors expressed on human leukocytes.
(Adapted from Baggioioli (1998))

4.1.4 Functional Groupings of Chemokines

Besides their structural criteria, chemokines are generally classified according to their functional grouping (Moser et al., 2004) as follows:

Inflammatory chemokines (e.g. CXCL1, CXCL2, CXCL3, CXCL5, CXCL8(IL-8), CXCL10, and CXCL11) (Zlotnik & Yoshie, 2012) are those chemokines that are upregulated and produced in response to infection or injury. Most of the inflammatory chemokines are clustered in chromosome 4 and chromosome 17. They are mainly involved in the recruitment and the migration of leukocytes into the infected or damaged site. Among the inflammatory chemokines are also the angiogenic chemokines (e.g. CXCL2)
which have an ELF (Glu-Leu-Arg) motif just before the first cysteine residue (Zlotnik & Yoshie, 2012). Their effects are mediated via the chemokine receptors CXCR1 and CXCR2 and promote the development of blood vessels (pro-angiogenic). Others (CXCL4, CXCL9, CXCL10) are non-ELR motif angiostatic chemokines (Kiefer & Siekmann, 2011) and they prevent the development of blood vessels (anti-angiogenic) (Mackay, 2001).

Figure 20: Homeostatic Chemokines. Adapted from (Zlotnik et al., 2011).

Homeostatic chemokines (e.g. CXCL12, CXCL13, CXCL14, CXCL19, CXCL20, CXCL21, CXCL25, and CXCL27) (Zlotnik et al., 2011) are expressed constitutively and restricted to specific cells or organs i.e. lymphoid, skin, small intestines, bone marrow, lung, spleen, and liver (Figure 20) (Zlotnik & Yoshie, 2012). They are located singly or in small clusters in different chromosomes. They are chemotactic for cells (subsets of lymphocytes and dendritic cells) that mediate acquired immunity and many of homeostatic chemokines are also found to play a pivotal role in the migration of cells during the normal development and maintenance of tissues and lymphoid organs (Moser et al., 2004; Zlotnik et al., 2006). The classification of chemokines is not mutually exclusive. Some inflammatory chemokines function as homeostatic whereas some homeostatic chemokines
function as inflammatory. These chemokines that overlap both fields and are called dual-function chemokines (e.g., CCL11, CCL17, CCL20, CCL22, XCL1, XCL2, CX3CL1).

The importance of all other chemokines, their receptors that participate in inflammatory processes, their ability to recruit distinct leukocytes subsets, and/or their involvement in other crucial processes as outlined above is acknowledged. This however lies outside the scope of this study which focuses on IL-8 and its roles in chronic urticaria.

4.2.0 Interleukin 8

Interleukin-8 (IL-8) (also known as CXCL 8) is a small (8kDa) soluble protein that belongs to the CXC chemokine subfamily (Rossi & Zlotnik, 2000). The human genes of the CXC chemokines are clustered on chromosome 4 (Baggiolini et al., 1994). For its biological effectiveness IL-8 binds to two cell surface G protein coupled receptors CXCR1 and CXCR2 (Baggiolini et al., 1994). CXCR1 and CXR2 are mainly expressed on neutrophils and they share 77% identical amino acids (Lotti & Maggi, 2013). These receptors are also found on monocytes, basophils, and eosinophils (Figure 19 ) but have a weaker response to IL-8 as compared to neutrophils(Baggiolini et al., 1994). The first member of the chemokine family to be discovered was the platelet factor 4 (now called CXCL4) (Mackay, 2001; Zlotnik & Yoshie, 2012) but IL-8 was the first chemokine to be characterised (Yoshimura et al., 1987). IL-8 is a multifunctional proinflammatory cytokine that is produced under conditions of inflammatory stimulation. It is primarily released from monocytes, macrophages, fibroblasts, keratinocytes, and endothelial cells (Ertugrul et al., 2013; Steiner et al., 2002) but also suggested that it could be secreted by lymphocytes, epithelial cells, and tumor cells (Ertugrul et al., 2013).

4.2.1 Interleukin 8 Expression

Expression of IL-8 is activated by pro-inflammatory cytokines like TNF-α and IL-1β (Lotti & Maggi, 2013) and also by pathogen derived signals such as lipopolysaccharides (LPS) on toll-like receptors (Ertugrul et al., 2013). IL-8 is mainly known to be a physiological initiator of the chemotactic migration of neutrophils. It participates in the activation and degranulation of neutrophils, chemoattracting them to the site of the infection (Ertugrul et al., 2013; Xie, 2001) Figure 21.
4.2.2 Interleukin 8 and Neutrophils

Chemotaxis is the movement of cells in response to chemotactic factors (chemokines bound to the tissue matrix). The movement is directional, and it corresponds to a gradient of increasing or decreasing concentration of chemotactic factors. It is a complex process that involves extracellular and intracellular signalling. The movement of neutrophils to sites of infection is guided by bacterial products such as formyl-Met-Leu-Phe (fMLP) (Foxman et al., 1997; Foxman et al., 1999) and most significantly by the host-derived IL-8 (Baggiolini, 1998; Baggiolini et al., 1997). IL-8 provides directional cues by establishing a concentration gradient and guides neutrophils in the underlying tissues. The neutrophils migrate in an increasing gradient direction and the migration is strongly dependent on the concentration of IL-8. It is reported that the mean IL-8 concentration rather than gradient steepness is the determining factor in controlling effective chemotaxis (Lin et al., 2004). It is also reported in the same study that the motility index (MI) and the effective chemotactic index (ECI) are strongly correlated in stable linear IL-8 gradients suggesting that effective chemotaxis is possibly strongly regulated by the mean IL-8 concentration dependent motility (Lin et al., 2004).

4.2.3 Interleukin 8 and Inflammation

During an inflammatory response, IL-8 travels through different compartments from its point of production and is taken up by venular endothelial cells and transcytosed to the luminal surface where it is immobilized on endothelial cell surface glycosaminoglycans (GAGs) for presentation to circulating neutrophils (Das et al., 2010; Mackay, 2001).

This role played by IL-8 is vital because neutrophils are sentinel cells of the innate immune system, known to be the principal cellular responders to acute inflammation (Looney & Matthay, 2009). Although IL-8 was originally identified as a cytokine that plays an important role as an activator and chemoattractant for neutrophils, its expression...
has since been found to be elevated in various human cancers that include melanoma (Huang et al., 2002), breast (Miller et al., 1998), renal (Slaton et al., 2001), gastric (Zhang et al., 2002), ovarian (Venkatakrishnan et al., 2000), pancreatic (Shi et al., 1999) and colorectal cancers (Li et al., 2001). Lately, IL-8 has also been seen to play a pivotal role in angiogenesis through activation of the vascular endothelial growth factor pathway (VEGF) (Dong & Zheng, 2015). In this pathway, IL-8 induces the VEGF expression on endothelial cells and the VEGF acts as an autocrine and paracrine factor on vascular endothelium. Lattanzio et al (2013) supposed that this interaction of IL-8 and VEGF seems to synergistically act to induce and maintain angiogenesis.

Several previous studies have provided evidence to support the concept that activated mast cells undergoing degranulation release some cytokines including IL-8 (Curto-Barredo et al., 2014; Zuberbier et al., 2014). In addition, other studies suggest TREM-1 enhances proinflammatory chemokine production (Belibasakis et al., 2014; Varanat et al., 2017). Kuai et al (2009) discovered that TREM-1 induces the production of multiple pro-inflammatory cytokines including IL-8. Wang et al (2012) reported that TREM-1 positively regulates the production of IL-8. Derive et al (2012) discovered that TREM-1 inhibition was linked to a decrease in the production of LPS- induced cytokines including IL-8. It is also speculated that microorganisms such as Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, and Aggregatibacter actinomycetemcomitans and the toxins they release in periodontal tissues stimulate the formation of IL-8 (Ertugrul et al., 2013; Mathur et al., 1996; Tsai et al., 1995).

As far as it is known, there are currently no studies investigating cytokine concentrations in the saliva of chronic urticaria patients. Defining this association would provide a valuable tool in identifying cases of chronic idiopathic urticaria associated with infection. The aim of this study is to determine and compare the levels of IL-8, sTREM-1, and IL-3 in the saliva of healthy subjects controls, and in patients with chronic urticaria.

This study tests the hypothesis that there are significantly higher salivary IL-8, sTREM-1, and IL-3 levels in chronic urticaria patients compared to normal control subjects and that IL-8 levels are correlated with salivary sTREM-1.

4.3. Method and Materials
There were no Spike/Recovery and Linearity experiments performed for salivary IL-8. It is assumed therefore that recovery of IL-8 in saliva is the same as for the more
complex matrix represented by serum and plasma and is in the acceptable recovery range of 80-120%. Assay Procedure for IL-8 and Statistical Analysis are described in detail in Chapter 2

4.4 Results
A calibration curve was included on each 96-well assay using the recombinant human IL-8 standard provided within the assay kit (R & D systems) Figure 22. The recombinant IL-8 standard was freshly prepared and further serially diluted to prepare the standard curve. The optical density was measured and presented as the mean value of the duplicates (Table 31). The curve was based on the 7 calibrator points of rh-IL-8 in the calibrator diluent, in the range of 15.625 to 1000 pg/ml (Table 31).

![IL-8 standard curve](image)

Figure 22: IL-8 standard curve
The standard curve was obtained by plotting concentration versus OD readings (Table 31) on log-log axes.

A seven-point standard curve using 2-fold serial dilutions in reagent diluent with a high standard of 1000pg/ml was recommended and used in this protocol.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (pg/ml)</th>
<th>Optical Density (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>1000</td>
<td>2.857</td>
</tr>
<tr>
<td>Standard 2</td>
<td>500</td>
<td>2.1975</td>
</tr>
<tr>
<td>Standard 3</td>
<td>250</td>
<td>1.825</td>
</tr>
<tr>
<td>Standard 4</td>
<td>125</td>
<td>1.25</td>
</tr>
<tr>
<td>Standard 5</td>
<td>62.5</td>
<td>0.798</td>
</tr>
<tr>
<td>Standard 6</td>
<td>31.25</td>
<td>0.4725</td>
</tr>
<tr>
<td>Standard 7</td>
<td>15.625</td>
<td>0.277</td>
</tr>
</tbody>
</table>

Table 31: Standards results of seven points using 2-fold serial dilutions

4.4.1 Spike/Recovery and Linearity Results for IL-8.

No spike/recovery and linearity tests were performed for IL-8. Although spike/ recovery and linearity experiments were not done with saliva for IL-8 measurements, the claim that recovery is 80-120% for all R&D Systems cytokine ELISA kits in plasma and serum is the same as 81-89% recovery for sTREM-1 and 97-106% for IL-3 in saliva. It is assumed therefore that recovery of IL-8 in saliva is the same as for the more complex matrix represented by serum and plasma and is also in the acceptable recovery range of 80-120%.

4.4.2 Patients’ UAS7 Score Results.

The UAS7 score results are shown in Chapter 3 and discussed in detail in Chapter 6.

4.4.3 Blood Tests Results for CRP, ESR, C3, C4, and Vitamin D

The blood results are shown in Chapter 3 and discussed in detail in Chapter 6.

4.4.4 Statistical Analysis of Salivary IL-8 Results

The Shapiro-Wilk test was used first to test whether the results were normally distributed. Data for salivary IL-8 concentrations in control samples, but not in samples from patients with urticarial, was normally distributed (Table 32), hence subsequent statistical analysis, therefore, used non-parametric tests. Mann-Whitney U test and the one-way ANOVA (Kruskal-Wallis test, with Dunn’s multiple comparisons) were used to assess the control-patient group comparisons, and p<0.05 values were considered statistically significant. Correlations among all assays were analysed by Spearman’s rank test, and p<0.01 values were considered as significant.
<table>
<thead>
<tr>
<th>Shapiro-Wilk test</th>
<th>Control IL-8</th>
<th>Urticaria IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>0.9440</td>
<td>0.8821</td>
</tr>
<tr>
<td>P value</td>
<td>0.1063</td>
<td>0.0006</td>
</tr>
<tr>
<td>Passed normality test (alpha=0.05)?</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
<td>***</td>
</tr>
</tbody>
</table>

Table 32: IL-8 levels in saliva of patients with urticaria and normal control subjects as determined by the Shapiro-Wilk normality test.

Figure 23: Salivary IL-8 in patients with chronic urticaria and control healthy subjects.

IL-8 was measured by ELISA in the saliva of patients (n=40) and healthy controls (n=31) that was collected using a salivette. Data is presented as individual values; the horizontal bar represents the median value. *; p<0.0001 (Mann-Whitney test).

IL-8 was significantly (p<0.0001) higher in the saliva of patients with urticaria, (232 pg/ml) than normal control values (127 pg/ml). The median (25th-75th percentile) value for salivary IL-8 in healthy controls was 127 pg/ml (58-164 pg/ml) and for patients with urticaria was 232.0 pg/ml (148.8-557 pg/ml). All values were above the LOD and there was no need to censor the data.
4.4.6 ROC Curve Analysis

In order to assess the performance of salivary cytokine analysis as a diagnostic test for CU, a receiver operating characteristic (ROC) curve over the range of possible cutpoints for the predictor variable was prepared for each analyte (Mandrekar, 2010a, 2010b).

The ROC curve shows the sensitivity and specificity of the results. It does this by comparing all the results in a group of patients with a group of controls.

<table>
<thead>
<tr>
<th>Area under the ROC curve</th>
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<tr>
<td>Area</td>
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<tr>
<td>Std. Error</td>
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<td>95% confidence interval</td>
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<td>P value</td>
<td>&lt;0.0001</td>
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<tr>
<td>Patients (Urticaria)</td>
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<td>Missing Patients</td>
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</tr>
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</table>

Table 33: Area under the ROC curve for IL-8

The ROC curve for salivary IL-8 (Figure 24) was significant, (p value<0.0001), area under the ROC curve 0.796, std. error 0.052, 95% confidence interval 0.694 to 0.898 Table 33. This suggests an 80% chance that IL-8 will correctly distinguish CU patients with suspected oral infection from normal.

Figure 24: ROC curve for salivary IL-8
4.4.7 Salivary IL-8 Data for Untreated Patients, Omalizumab Treated, and Healthy Controls.

The patients were further subdivided into naïve (untreated patients), omalizumab treated (OMA) and healthy control groups and salivary cytokine data analysed by non-parametric, unpaired, analysis using the one-way ANOVA (Kruskal-Wallis test, with Dunns multiple comparisons), Table 34. Salivary IL-8 was significantly higher in both groups of patients, naïve (untreated) (p=0.0184) and those treated with OMA (p=0.0001) Table 35. Omalizumab appears to have no effects on IL-8 Figure 25.

Figure 25: Salivary IL-8 in naïve, untreated, omalizumab treated patients with urticaria and control healthy subjects.
The graph shows individual data points and median values.
Multiple Comparisons

<p>| | | | | |</p>
<table>
<thead>
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<td>Significant?</td>
<td>Summary</td>
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</tr>
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<td>*</td>
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<tr>
<td>Column A vs. Column C</td>
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<td>0.0001</td>
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Test details

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<th>n2</th>
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<td>-17.07</td>
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<td>46.32</td>
<td>-22.14</td>
<td>31</td>
<td>25</td>
<td>4.048</td>
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</table>

Table 34: Kruskal-Wallis test, with Dunns multiple comparisons of IL-8 in saliva of naïve, untreated, CU (column B), Omalizumab treated CU (column C) and healthy control (column A).

4.4.8 ROC Curve Analysis of Untreated Patients vs Healthy Controls

The ROC curve for salivary IL-8 was again significant for untreated patients vs healthy controls Table 35. The ROC curve for salivary IL-8 was again significant, (p value<0.0108) for untreated vs healthy, area under the ROC curve 0.7396, std. error 0.09093, 95% confidence interval 0.5614 to 0.9179 Table 35 and Figure 26. This suggests a 74% chance that IL-8 will correctly distinguish CU patients with suspected oral infection from normal.

<table>
<thead>
<tr>
<th>Area under the ROC curve</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>0.7396</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.09093</td>
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<tr>
<td>95% confidence interval</td>
<td>0.5614 to 0.9179</td>
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<tr>
<td>P value</td>
<td>0.0108</td>
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<tr>
<td>Controls (Healthy control)</td>
<td>31</td>
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<tr>
<td>Patients (CU untreated)</td>
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<td>Missing Controls</td>
<td>1</td>
</tr>
<tr>
<td>Missing Patients</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 35: Area under the ROC curve for IL-8 in untreated (naïve), omalizumab treated patients with urticaria and control healthy subjects.
Figure 26: The ROC curve for salivary IL-8 for untreated patients vs healthy controls

A cut off value for IL-8 that gave the best combination of sensitivity and specificity was chosen from the raw data, calculated from the GraphPad prism Table 36. 168.5 pg/ml was the cut-off value chosen for salivary IL-8 and it shows that salivary IL-8 has a specificity of 80% and sensitivity of 70%. The likelihood ratio (LR) was 3.6, indicating that the test is more likely to be positive in disease. It was also calculated using the GraphPad prism.

Table 36: Sensitivity, Specificity and Likelihood ratio calculated from IL-8 raw data

4.4.9 Negative Predictive Value and Positive Predictive Value of IL-8

The negative predictive value (NPV) and the positive predictive value (PPV) were also calculated based on a projected point prevalence in the population for CU of 1% (Powell et al., 2015). The PPV is low, only 3.4% but the negative predictive value (NPV) is high (99.6%).
4.5 Discussion

4.5.1 Summary of IL-8 Key Findings

The present study was carried out to investigate the potential usefulness of salivary cytokines as biomarkers in chronic urticaria. As far as it is known, this is the first study to compare total levels of IL-8 in the saliva of healthy subjects and chronic urticaria patients. Significantly higher levels (p < 0.001) were found in chronic urticaria patients (median 232.0 pg/ml) compared to healthy controls (median 127 pg/ml). These elevated levels of IL-8 suggest the persistent presence of oral bacterial infection in CU patients. The ROC curve for salivary IL-8 was significant, (p value<0.001), and the area under the ROC curve (AUC) was 0.796. This value suggests that IL-8 is potentially a good diagnostic marker for CU with suspected oral infections, bordering excellent area under the curve (AUC 0.8-0.9) (Mandrekar, 2010a). This suggests an 80% chance that IL-8 will correctly distinguish CU patients with suspected oral infection from normal. The patients were further subdivided into naïve (untreated patients), omalizumab treated (OMA), and healthy control groups and salivary cytokine data analysed by non-parametric, unpaired, analysis using the one-way ANOVA (Kruskal-Wallis test, with Dunns multiple comparisons). Salivary IL-8 was significantly higher in both groups of patients, naïve (untreated) (p=0.0184) and those treated with omalizumab (p=0.0001). Omalizumab appears to have no effects on IL-8. The ROC curve for salivary IL-8 was again significant, (p value<0.0108) for untreated vs healthy, area under the ROC curve 0.7396, std. error 0.09093, 95% confidence interval 0.5614 to 0.9179.

The cut-off value for salivary IL-8 of 168.5 pg/ml showed that salivary IL-8 has a specificity of 80% and sensitivity of 70%, and is comparable to a previous ASST study (Sabroe et al., 1999). The likelihood ratio is therefore the same (LR=3.5), that is the test is more likely in disease, but the ELISA test is possibly more quantitative and less subjective.

The ROC analysis indicates salivary IL-8 could be a diagnostic marker for CU patients suspected of oral infection, but the positive predictive value (PPV) will be low and NPV will be high for CU which has a low prevalence. The PPV and the NPV are influenced by the prevalence of a disease in a population that is being tested. In a high prevalence setting, it is more likely that the persons who test positive truly have the disease than if the test is performed in a population with low prevalence. The negative predictive value (NPV) and the positive predictive value (PPV) in this study were calculated based on a projected point
prevalence in the population for CU of 1%. The British Society for Allergy and Clinical Immunology guidelines for the management of chronic urticaria and angioedema (BSACI guidelines) state that the lifetime prevalence of chronic urticaria is 0.5−1% (Powell et al., 2015). Although rarely life-threatening, CU significantly reduces the quality of life.

If the point prevalence is only 1% then the PPV is low, only 3.4%, but the negative predictive value (NPV) is high (99.6%) and therefore a low IL-8 value would rule out CU patients suspected of having an oral infection, and the value of the test is largely in the NPV. CU diagnosis therefore could not be based on a single salivary IL-8 but would need other confirmatory tests.

4.5.2 Potential Impact of IL-8 on Clinical Practice

The cytokine data indicates new opportunities for the analysis of salivary IL-8 and its development to a new diagnostic biomarker in CU patients. It has also provided a new perspective on the functioning of the immune system in these patients. As far as it is known very few clinicians take their time to examine the mouths and teeth of patients with chronic urticaria as well as taking a saliva sample for testing when these patients come for their clinical appointments. The IL-8 results of this study will help to inform the clinicians to always perform an oral examination and take a saliva sample from chronic urticaria patients when they visit the allergy clinic and other primary care clinics for their routine allergy appointments. The IL-8 data has the potential to indicate that oral bacterial infection is a tractable trigger for chronic urticaria even though no infection analysis was done in the patients in this study. Increased levels of salivary IL-8 have been obtained in previous studies whose patients had periodontitis but no CU (Mathur et al., 1996; Tsai et al., 1995), hence making it difficult to make a comparison between this study and the previous studies. Although previous reports have shown a trend of elevated salivary IL-8 in patients with oral bacterial infections, some studies have reported higher IL-8 in healthy control groups (Chung et al., 1997; Ozmeriç et al., 1998). Additionally, a recent review posits that mast cell activation by IgE alone and other non-bacterial stimuli can account for increased IL-8 in humans biological samples (Bradding & Arthur, 2016).

The findings in this study confirm the capacity of and IL-8 to be helpful as a diagnostic marker in CU patients. There are several potential advantages of using salivary IL-8 in CU patients. The patient and financial benefits of this new procedure could be enormous. The technique used for IL-8 determination is relatively quick and easy, non-invasive,
inexpensive, and can be performed for small batches of specimens. Early diagnosis of bacterial infections remains challenging particularly in patients with a multiplicity of clinical presentations. The specific involvement of IL-8 solely in cases of bacterial infections would be extremely helpful to discriminate between chronic urticaria patients with and without infection. This is highly desirable because prompt and effective antibiotic treatment is crucial in the treatment of chronic urticaria patients with oral bacterial infections (Tadros et al., 2018).

In contrast to the IL-8 levels, the levels of the routine blood tests (ESR, CRP, C3C4, and Vitamin D) for CU patients at UHS were normal. The routine blood tests results are discussed in detail in chapter 6.

The slow recruitment of naïve patients coupled with research time constraints resulted in the study focusing on medicated CU patients. Most of the patients (n=27) in this study were on Omalizumab treatment and a few were naïve patients (n=15) with no record of treatment. It was not known for how long these patients had been on omalizumab. Omalizumab is discussed in detail in Chapter 2 and Chapter 6.

Bacterial culture is the gold standard method for detection and isolation of microorganisms in body fluids and airways of patients suspected of bacterial infections (Baker et al., 1996; Gibot, 2006, 2009; Gibot, Cravoisy, et al., 2004; Loftus et al., 2016; Rello et al., 2014; Wunderink, 1998). Regrettably, there were no culture tests done in this study. Bacterial culture is discussed in detail in chapter 6.

4.5.3 Limitations

A limitation to this study is the small number of participants enrolled. The CLSI EP12-A2 guideline ("EP12-A2 User protocol for evaluation of qualitative test performance; approved guideline," 2008) recommends that as a minimum testing should continue until results from at least 50 positive specimens are obtained and that at least 50 negative specimens using the comparative method should be obtained to determine the specificity of the candidate method. However, although this was the aim of this postgraduate study, it was not possible in the time frame and the study finally recruited 43 patients and 34 controls. Moreover, there were no bacterial cultures done and salivary CRP was not tested. ESR is a test that can only be done using full blood. Future work would require many participants, more salivary tests such as CRP and PCT.
4.5.4 Conclusion
A significant increase was observed in the levels of salivary IL-8 in the saliva of CU patients relative to healthy control subjects. These elevated levels of salivary IL-8 suggest the persistent presence of oral bacterial infection in CU patients. IL-8 may play a key role in the host response to infection in the oral cavities of CU patients.

Infection in the oral cavity of CU is one among many factors that trigger CU hence identification of IL-8 may be very important in the prognoses of CU. In contrast, the levels of the routine blood tests (ESR, CRP, FBC, C3C4, and Vitamin D) were normal. Based on the results of this study, there is increasing evidence that salivary IL-8 may represent the main pro-inflammatory chemokine related to bacterial infections. It is therefore concluded that IL-8 together with other biomarkers will have a progressive important clinical role to identify CU patients with oral bacterial infections. Using it with other biomarkers will help to accelerate diagnosis as well as start a specific therapy and monitor the course of bacterial infections in CU patients.
Chapter 5: Interleukin 3 (IL-3)

Mast cells and basophils are important in the pathology of chronic urticaria. IL-3 is an important cytokine that plays a central role of modulating these cells expansion and activity in allergic responses (Marone et al., 2014; Varricchi et al., 2018). It increases histamine release from basophils and mast cells (Curto-Barredo et al., 2014; Ferrer et al., 2003; Shimizu et al., 2008). The relevance of IL-3 in the development and progression of chronic urticaria as has been uncovered in previous studies (Ferrer et al., 2003; Shimizu et al., 2008; Ye et al., 2014) necessitated its inclusion in this study. More roles of IL-3 are discussed in the paragraphs below.

5.1.0 Cytokines

Interleukins are cytokines that play an important role in communication between inflammatory cells following the activation of the immune system. Cytokines are small soluble proteins that mediate and regulate local, systemic, and inflammatory responses and they participate in many biological events (Ertugrul et al., 2013). They regulate the generation, survival, and function of cells by binding to cell surface receptors (Voehringer, 2012). Cytokines perform critical roles in the control of fundamental pathways of the immune system (Labidi et al., 2010), by engaging in the communication between cells of the immune system. They are classified according to their functional grouping as: (a) mediators and regulators of innate immunity which act on endothelial cells and leukocytes to stimulate an early innate response, (b) mediators and regulators of adaptive immunity which act on lymphocytes to stimulate and regulate adaptive responses to specific infections, (c) stimulators of haematopoiesis which act on the bone marrow to stimulate growth and differentiation of leukocytes and lymphocytes. Cytokines are involved in both cellular and antibody-mediated immunity. They direct the growth, development, maturation, activation, and life span of immune cells. They play a major role in the inflammatory and immune responses and several of them display inflammatory and other anti-inflammatory responses. Elevated levels of certain cytokines have been shown to associate with the diagnosis and prognosis of a variety of diseases (Lugos et al., 2019), including haematological cancers such Hodgkin and Non-Hodgkin lymphomas (Casasnovas et al., 2007; Pedersen et al., 2005; Salven et al., 2000). Hence, this study assesses the expression of IL-3 in the saliva of CU patients.
5.1.1 Interleukin-3

Interleukin-3 (IL-3) is a haematopoietic factor that belongs to the β common (βc/CD131) family of cytokines (Borriello et al., 2019; Hercus et al., 2018). It is known to be a pleiotropic growth factor that stimulates the proliferation, differentiation, and survival of pluripotent haematopoietic stem cells (Blalock et al., 1999; Farrar et al., 1989; Lindemann & Mertelsmann, 1995; Nishinakamura et al., 1996). The βc cytokines are predominantly expressed by activated T cells, but many other cell types such as macrophages, mast cells, epithelial cells, eosinophils, basophils, endothelial cells, monocytes, B cells, fibroblasts, and keratinocytes have been reported to express selected βc cytokines Figure 27 (Hercus et al., 2018).

Granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-5 (IL-5), and interleukin-like protein (KK34) are also part of the β common family of cytokines, and they use βc as their key signalling receptor subunit (Borriello et al., 2019; Hercus et al., 2018). The gene for IL-3 has been mapped to human chromosome 5 and is closely linked to the genes for IL-4, IL-5, and GM-CSF (van Leeuwen et al., 1989) The gene spans 2.7kb.
and has 5 exons and composed of 152 amino acids. In early studies, IL-3 was initially characterised by its capability to cause/promote the production of the enzyme 20-\(\alpha\)-hydroxysteroid dehydrogenase in mice splenocytes (Ihle et al., 1981; Ihle et al., 1985). Further studies revealed the growth stimulatory activities of IL-3 which were being studied by different investigators under different names, including Thy-1 inducing factor (Ihle et al., 1981), persisting cell-stimulating factor (PSF) (Schrader et al., 1981), mast cell growth factor (MCGF) (Nabel et al., 1981), haematopoietic cell growth factor (HCGF) (Bazill et al., 1983), histamine-producing cell-stimulating factor (Dy et al., 1981), multi-colony stimulating factor (multi-CSF) (Schrader & Clark-Lewis, 1982) and burst promoting activity (BPA) (Ihle et al., 1983). All these stimulatory growth activities were subsequently identified as the same protein and renamed IL-3 (Blalock et al., 1999).

### 5.1.2 Interleukin -3 Expression

IL-3 is expressed by several haematopoietic and non-haematopoietic cell types including human T cells (Niemeyer et al., 1989; Van Straaten et al., 1994), natural killer cells (Cuturi et al., 1989), mast cells (Möller et al., 1998), epithelial cells (Dalloul et al., 1991), stromal cells (Gibson et al., 1995), murine keratinocytes (Peterseim et al., 1993), neurons (Farrar et al., 1989) and astrocytes (Farrar et al., 1989). Although it is expressed by many cell types, the main source of IL-3 is suggested to be T lymphocytes upon T cell receptor (TCR) activation and under Th2 polarizing conditions such as allergic inflammation and parasitic infestation (Kim et al., 2010; Leyva-Castillo et al., 2013; Ohta et al., 2017). However, there is also increasing evidence that IL-3 is mainly produced by basophils when they are activated via Fc\(\varepsilon\)RI or the C3a/C5a receptors (Schroeder et al., 2009). Biologically inactive IL-3 is thought to be stored in the extracellular matrix as complexes with proteoglycans although the mechanism for activation is unknown.
Blaock et al (1999) proposed the IL-3 activation pathway. The pathway indicates that diacylglycerol (DAG) and Ca2+ activate PKC. The activation of these two molecules is then followed by the activation of calmodulin, calcineurin, NF-kB, and NF-AT. In this pathway, NF-kB can also be activated by NIK. The activation of the transcription factors leads to the activation and expression of IL-3. Immunosuppressive drugs such as CsA, FK506, and DSG can inhibit the activation of IL-3 by blocking the activation of the transcription factors.

PKC; protein kinase c, NF-kB; nuclear factor kappa B, NF-AT; nuclear factor of activated T-cells, NIK; NF-kB-inducing kinase, CsA; cyclosporin A, FK506; Tacrolimus, DSG; deoxyspergualin Adapted from Blalock et al 1999.

5.1.3 Interleukin-3 Activation Pathway

The IL-3 activation pathway (Figure 28) shows the potential usefulness of CsA and other drugs in the treatment of CU patients. Treatment of CU patients is discussed more in the Treatment/Management section in Chapter 1. IL-3 exerts its function through a complex of a heterodimeric common beta chain (βc) receptor and the high affinity IL-3R alpha chain (IL-3Rα) Cluster of Differentiation 123 (CD123) (Borriello et al., 2019). This interaction results in the activation of Janus kinases (JAKs) leading to tyrosine residues

Figure 28: Activation of IL-3 expression.
phosphorylation. Tyrosine residues are present on the (β_c) receptor and signal transducer & activator of transcription (STAT) proteins (Voehringer, 2012).

5.1.4 Interleukin-3 and Inflammation

IL-3 coded blue in Figure 28 has actions and relevant biological significance on the widest range of cell types. IL-3 is now known to play a key role in both immune and neurodevelopmental systems. IL-3 has been found to advance the generation and differentiation of myeloid cells during infection and inflammation (Lantz et al., 1998; Weber et al., 2015). Also, elevated IL-3 levels have been observed in inflammation especially in the late phase response (Falcone et al., 2000; Shimizu et al., 2008). Also, IL-3 is released as a mediator during basophils degranulation. Basophils have IL-3 cell surface receptors CD123, and its actions are mediated via CD123. Basophils are both the target and source of IL-3, which also act in an autocrine manner to modulate basophil survival and cytokine production (Rignault-Bricard et al., 2018; Schroeder et al., 2009). The various effects of IL-3 on human basophils also include development (Cozon et al., 1999), growth (Vilariño et al., 2005), migration (Iikura et al., 2004), recruitment (Leyva-Castillo et al., 2013) apoptosis inhibition, and more importantly for the purpose of this study, the release of histamine. IL-3 plays a central role in modulating basophil and mast cell expansion and activity in type 2 allergic responses (Marone et al., 2014; Varricchi et al., 2018). Th2 lymphocytes secrete IL-3 that expand and recruit basophils which in turn reinforce Th2 polarization (Kim et al., 2010; Leyva-Castillo et al., 2013; Ohta et al., 2017).

IL-3 also plays a crucial role in the augmented secretion of cytokines IL-4 and IL-13 (Borriello et al., 2015). In addition, IL-3 is known to activate monocytes in combination with IL-4, promoting monocytes differentiation into dendritic cells (DCs) (Ebner et al., 2002; Sallusto & Lanzavecchia, 1994).

Also, IL-3 boosts the expression of chemokine CCL17 alone or in combination with IL-4 (Borriello et al., 2016; Borriello et al., 2015; Hsu et al., 2018). CCL17 is a small cytokine that belongs to the CC chemokine family which binds and induces chemotaxis in T cells.

IL-3 has been reported to contribute to diseases in humans, either directly or indirectly. CD123, the IL-3 specific receptor is highly expressed in haematological malignancies such as acute myeloid leukaemia (AML) (Jin et al., 2009; Jordan et al., 2000; Testa et al., 2002; Vergez et al., 2011) and chronic myeloid leukaemia (CML) (Nievergall et al., 2014). IL-3 is also suggested to play a central role in the pathogenesis of sepsis and its potential
usefulness as a diagnostic marker of sepsis (Borriello et al., 2019). Singer et al (2016) define sepsis as a life-threatening organ dysfunction caused by a dysregulated host response to infection. IL-3 has also been implicated in promoting emergency myelopoiesis and cytokine storm (Rauch et al., 2012; Weber et al., 2015). Elevated blood IL-3 levels of septic patients are associated with increased mortality (Weber et al., 2015). Additionally, elevated blood levels of IL-3 are associated with high organ failure rates, therefore enhancing the need to implement the use of IL-3 as a biomarker of sepsis in the clinical setting (Min et al., 2018). IL-3 is suspected to be involved to a lesser extent in autoimmune diseases (Borriello et al., 2019). Controversially, it has been reported that IL-3 either attenuates (Srivastava et al., 2011) or exacerbates arthritis symptoms (Brühl et al., 2009). Eosinophils are the hallmark cells of allergic inflammation (Rosenberg et al., 2013) and are mainly activated by IL-5, however, several studies have shown that IL-3 activates eosinophils as well (Esnault et al., 2015; Soman et al., 2017). IL-3 has been implicated in promoting the extramedullary haematopoiesis and differentiation of monocytes that infiltrate and aggravate atherosclerotic lesions (Robbins et al., 2012). IL-3 stimulates macrophage production of matrix metalloprotease 12 (MMP12) which in turn enhances the development of chemically induced thoracic aortic aneurism and dissection (Liu et al., 2018). IL-3 has been investigated in cancer therapy as it is suggested to be a survival factor in several haematopoietic malignancies. As such IL-3 or its alpha chain receptor, CD123 has been noted as therapeutic targets in AML where CD123 expression on cancer cells correlates with patient survival (Jordan et al., 2000; Testa et al., 2002; Vergez et al., 2011).

5.1.5 Interleukin -3 and Chronic Urticaria
Relevant to this study is the association of IL-3 with chronic urticaria. IL-3 is associated with the upregulation of CD203c as well as CD69 expression that results in basophil activation and histamine release. CD203c upregulation is suggested to be mediated by either FcεRI or IL-3 with increased CD203c expression on basophils known to be associated with exacerbated chronic urticaria symptoms (Ye et al., 2014). In a study by Ferrer et al, (2003) basophils primed with IL-3 had a histamine release of at least 15% more than those tested without priming. However, the study was based on the basophil activation test (BAT) assay. Autocrine IL-3 upregulation of mast cell survival in the absence of antigen has been reported by Shimizu et al (2008) as instrumental in the exacerbation of chronic urticaria symptoms. Published data indicate that IL-3 is released as
a mediator by activated mast cells undergoing degranulation (Curto-Barredo et al., 2014; Zuberbier et al., 2014). The role of IL-3 and histamine in CU is described in detail in Chapter 1.

The current study assesses the expression of IL-3 in saliva in CU patients. As far as it is known, there are currently no studies investigating IL-3 concentrations in the saliva of chronic urticaria patients. Previous studies used different methods to measure IL-3 in skin lesions of CU patients (Hermes et al., 1999) and in the blood (Ferrer et al., 2003; Ye et al., 2014). See Chapter 1 and 2 for the choice of using saliva and the ELISA technique in this study.

This study aims to determine and compare the levels of IL-3 in the saliva of health subjects’ controls and patients with chronic urticaria.

This study tests the hypothesis that there are significantly higher salivary IL-3 levels in chronic urticaria patients compared to normal control subjects.

5.2 Method and Materials
Sample and control spike preparation, assay procedure and statistical analysis for IL-3 are described in detail in Chapter 2

5.3 Results

5.3.1 Spike/Recovery and Linearity Results for IL-3
A calibration curve was included on each 96-well assay using the recombinant human IL-3 standard provided within the assay kit (R & D systems). The rh IL-3 standard was prepared freshly every day and further serially diluted to prepare the standard curve. The optical density was measured and presented as the mean value of the duplicates (Table 37). The curve was based on the 7 calibrator points of rh-IL-3 in the calibrator diluent, in the range of 31,3 to 2000 pg/ml (Table 37).

The back-calculated concentration of the calibration standards in the range of 31.3-2000pg/ml met the acceptance criteria of mean accuracy within 80-120 % and precision (% CV) less than 10%. The IL-3 ELISA test reference curve was generated, using the log-log plot (Figure 29), with a good curve regression correlation R²=1. One representative out of the 6 different repeats is shown below.
Figure 29: IL-3 standard curve from the data in Table 38, plotted on log-log axes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (pg/ml)</th>
<th>Optical Density (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>2000</td>
<td>3.017</td>
</tr>
<tr>
<td>Standard 2</td>
<td>1000</td>
<td>1.812</td>
</tr>
<tr>
<td>Standard 3</td>
<td>500</td>
<td>0.945</td>
</tr>
<tr>
<td>Standard 4</td>
<td>250</td>
<td>0.500</td>
</tr>
<tr>
<td>Standard 5</td>
<td>125</td>
<td>0.258</td>
</tr>
<tr>
<td>Standard 6</td>
<td>63.5</td>
<td>0.127</td>
</tr>
<tr>
<td>Standard 7</td>
<td>31.3</td>
<td>0.065</td>
</tr>
</tbody>
</table>

Table 37: IL-3 Standards results of seven points using 2-fold serial dilutions.

5.3.2 Spike/Recovery Results

Spike/recovery and linearity experiments were repeated over 6 runs using different samples. These spike/recovery experiments were run on different days. Results for spike control and spike sample (donor) of salivary IL-3 analysis (Table 38 and 39) show % recovery. Successful recovery is when the mean % recovery obtained lies between 80-120% (Tiwari & Tiwari, 2010). A good % recovery was observed for 400pg/ml spiked control with a mean of 107±3.6% (range 101-120%) (Table 38) and similarly, a good % recovery was also observed for 400pg/ml spiked sample (healthy donor) with a mean of 100±7.3% (range 85-124%) (Table 39).
<table>
<thead>
<tr>
<th>ELISA RUN</th>
<th>SAMPLE</th>
<th>Protease Inhibitor used Y/N</th>
<th>% Recovery 1:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 1</td>
<td>N</td>
<td>101</td>
</tr>
<tr>
<td>2</td>
<td>Control 2</td>
<td>N</td>
<td>108</td>
</tr>
<tr>
<td>3</td>
<td>Control 3</td>
<td>N</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Control 4</td>
<td>N</td>
<td>104</td>
</tr>
<tr>
<td>5</td>
<td>Control 5</td>
<td>N</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td><strong>Mean± SEM</strong></td>
<td></td>
<td><strong>107±3.6</strong></td>
</tr>
</tbody>
</table>

Table 38: ELISA spike and recovery results of IL-3 spike controls (calibrator diluent).

<table>
<thead>
<tr>
<th>ELISA RUN</th>
<th>SAMPLE</th>
<th>Protease Inhibitor used Y/N</th>
<th>% Recovery 1:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Donor 1</td>
<td>Y</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>Donor 2</td>
<td>Y</td>
<td>116</td>
</tr>
<tr>
<td>3</td>
<td>Donor 3</td>
<td>Y</td>
<td>81</td>
</tr>
<tr>
<td>4</td>
<td>Donor 4</td>
<td>Y</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>Donor 5</td>
<td>Y</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>Donor 6</td>
<td>Y</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td><strong>Mean± SEM</strong></td>
<td></td>
<td><strong>100±7.3</strong></td>
</tr>
</tbody>
</table>

Table 39: ELISA spike and recovery results of IL-3 spike samples (healthy donor saliva)

**5.3.3 Linearity Results**

Good linearity in the concentration measured was observed at all dilutions for spike controls using 400 pg/ml except for the 1:8 dilution of control 4 and 5 and 1: 4 dilution of control 4. Mean %recovery at 200 pg/ml (1:2) spiked control was 99±3.6% (range 95-107%); mean %recovery at 100 pg/ml (1:4) spiked control was 93±5.6 % (range 73-106%); and mean %recovery 50 pg (1:8) spiked control was 79±11.1 % (range 44-104) (Table 40).

Good linearity was seen at all dilutions for all spiked healthy donor samples using 400 pg/ml except for the 1:8 dilution of donor 1,4, 5and 6: 1:4 dilution of donor 5. Mean %recovery at 200pg/ml (1:2) spiked donor sample was 108±5.1% (range 88-114%); mean %recovery at 100pg/ml (1:4) spiked donor sample was 104±9.6 % (range 62-124%); mean %recovery at 50pg (1:8) spiked donor sample was 91±15.3 % (range 44-144%) (Table 41).

Successful linearity was seen at 1:2 dilution for all samples. Overall, linearity appears to be lost as the samples were diluted down to 1:8 after spiking the calibrator diluent and donors with 400pg/ml (Tables 40 and 41). This loss of linearity was observed at 4 different
concentration levels and could be associated with either a technical error (random error) or systematic error. In the event of small samples or high results, a decision was made to dilute the test samples 1:2 to obtain valid results.

<table>
<thead>
<tr>
<th>ELISA Run</th>
<th>Sample Used</th>
<th>PI Used</th>
<th>% Recovery 1:0 400pg/ml</th>
<th>% Recovery 1:2 200pg/ml</th>
<th>% Recovery 1:4 100pg/ml</th>
<th>% Recovery 1:8 50pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 1</td>
<td>N</td>
<td>101</td>
<td>107</td>
<td>106</td>
<td>104</td>
</tr>
<tr>
<td>2</td>
<td>Control 2</td>
<td>N</td>
<td>108</td>
<td>95</td>
<td>94</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>Control 3</td>
<td>N</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>Control 4</td>
<td>N</td>
<td>104</td>
<td>88</td>
<td>73</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>Control 5</td>
<td>N</td>
<td>120</td>
<td>107</td>
<td>92</td>
<td>66</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td></td>
<td></td>
<td>107±3.6</td>
<td>99±3.6</td>
<td>93±5.6</td>
<td>79±11.1</td>
</tr>
</tbody>
</table>

Table 40: Linearity of recombinant IL-3 in calibrator diluent

<table>
<thead>
<tr>
<th>Run</th>
<th>Sample Used</th>
<th>PI Used</th>
<th>% Recovery 1:0 400pg/ml</th>
<th>% Recovery 1:2 200pg/ml</th>
<th>% Recovery 1:4 100pg/ml</th>
<th>% Recovery 1:8 50pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Donor 1</td>
<td>Y</td>
<td>85</td>
<td>98</td>
<td>124</td>
<td>144</td>
</tr>
<tr>
<td>2</td>
<td>Donor 2</td>
<td>Y</td>
<td>116</td>
<td>114</td>
<td>108</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>Donor 3</td>
<td>Y</td>
<td>81</td>
<td>109</td>
<td>126</td>
<td>126</td>
</tr>
<tr>
<td>4</td>
<td>Donor 4</td>
<td>Y</td>
<td>105</td>
<td>122</td>
<td>95</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>Donor 5</td>
<td>Y</td>
<td>88</td>
<td>88</td>
<td>62</td>
<td>44</td>
</tr>
<tr>
<td>6</td>
<td>Donor 6</td>
<td>Y</td>
<td>124</td>
<td>114</td>
<td>111</td>
<td>70</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td></td>
<td></td>
<td>100±7.3</td>
<td>108±5.1</td>
<td>104±9.6</td>
<td>91±15.3</td>
</tr>
</tbody>
</table>

Table 41: Linearity of recombinant IL-3 in donor saliva samples

5.3.4 Accuracy and Precision

Accuracy is the measure of how close the experimental value is to the true value. Accuracy calculates the deviation of the measured results against the true value.

Precision is the measure of how close data values are to each other for several measurements under the same analytical conditions and it assesses repeatability and calculates the relative standard deviation (Andreasson et al., 2015).

IL-3 ELISA for control (calibrator diluent) and a healthy donor was performed multiple times and the IL-3 concentrations were measured (Table 42 and Table 43). 400pg/ml rh-IL-3 was employed and further two-fold serially diluted. The precision of rh-IL-3
determination was carried out by the mean CV calculations. For accuracy, different rh-IL-3 concentrations were added to calibrator diluent controls and concentrations were measured.

Accuracy of the investigated IL-3 test was determined by standard addition comprising serial dilutions of the reference standard (recombinant human IL-3) in calibrator diluent and healthy donor saliva. The overall %recovery showed high accuracy of IL-3 detection in the calibrator diluent (with an exception at 50pg/ml of calibrator diluent control) and healthy donor saliva controls (Tables 42 and 43). Also, the precision of the investigated IL-3 test was determined by standard addition comprising serial dilutions of the reference standard (recombinant human IL-3) in calibrator diluent. No precision was performed on donor saliva samples because they were from different donors.

Precision should always be estimated from the same experiment in combination with replicate values (several measurements of the same sample under the same conditions) of several independent experiments following identical procedures (Hasibeder et al., 2015). The IL-3 test at 400pg/ml and 200pg/ml concentrations showed high precision, not exceeding a coefficient of variation of 10%, with low precision at 100pg/ml and 50pg/ml in the calibrator diluent controls (Table 42).

<table>
<thead>
<tr>
<th>Sample</th>
<th>1:0 (400pg/ml)</th>
<th>1:2 (200pg/ml)</th>
<th>1:4 (100pg/ml)</th>
<th>1:8 (50pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>403</td>
<td>213</td>
<td>106</td>
<td>52</td>
</tr>
<tr>
<td>Control 2</td>
<td>431</td>
<td>190</td>
<td>94</td>
<td>40</td>
</tr>
<tr>
<td>Control 3</td>
<td>400</td>
<td>200</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Control 4</td>
<td>416</td>
<td>176</td>
<td>73</td>
<td>22</td>
</tr>
<tr>
<td>Control 5</td>
<td>479</td>
<td>213</td>
<td>92</td>
<td>33</td>
</tr>
<tr>
<td>Mean+3SD</td>
<td>426+32</td>
<td>198+14</td>
<td>93+12</td>
<td>37+13</td>
</tr>
<tr>
<td>CV</td>
<td>8%</td>
<td>7%</td>
<td>13%</td>
<td>35%</td>
</tr>
<tr>
<td>Accuracy %</td>
<td>107+3.6</td>
<td>99+3.6</td>
<td>93+5.6</td>
<td>74+11.1</td>
</tr>
</tbody>
</table>

Table 42: Precision and accuracy for controls (calibrator diluent).
Table 43: Accuracy for healthy donor saliva samples

The IL-3 premade commercial QC materials (low, medium, and high) were also used to determine the accuracy and precision of the IL-3 test. Inter-assay and intra-assay accuracy and precision were assessed using four independent determinations of each QC level over 4 runs and one run respectively (Tables 44 and 45).

The three quality control materials were supplied by R&D Systems and were designed for cell supernatants, not saliva. Expected ranges and target values were provided as shown in Tables 44 and 45. Using the acceptable ranges provided by R&D systems designed for cell supernatants, all mean results for IL-3 were within the assay range. The inter-assay accuracy was good at each QC level, in the range of 110% to 112% (Table 33). The inter-assay precision was low with CVs exceeding 10% and was within the range of 16% to 23% at each QC level (Table 44). The intra-assay accuracy was good at each QC level, in the range of 89% to 95%. The intra-assay precision was excellent and was within the range of 1% to 4% (Table 45).

<table>
<thead>
<tr>
<th>QC</th>
<th>Range pg/ml</th>
<th>Target</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
<th>Mean+/sd</th>
<th>% Accuracy</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>128-176</td>
<td>152</td>
<td>151</td>
<td>184</td>
<td>123</td>
<td>212</td>
<td>168+/38</td>
<td>111</td>
<td>23%</td>
</tr>
<tr>
<td>Medium</td>
<td>453-575</td>
<td>514</td>
<td>587</td>
<td>574</td>
<td>436</td>
<td>654</td>
<td>563+/91</td>
<td>110</td>
<td>16%</td>
</tr>
<tr>
<td>High</td>
<td>962-1174</td>
<td>1038</td>
<td>1269</td>
<td>1165</td>
<td>886</td>
<td>1323</td>
<td>1161+/194</td>
<td>112</td>
<td>17%</td>
</tr>
</tbody>
</table>

Table 44: Inter-assay precision using Low, Medium, and High QC

QC samples were analysed over 4 runs for inter-assay precision.
<table>
<thead>
<tr>
<th>QC</th>
<th>Range pg/ml</th>
<th>Target</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Mean+-/sd</th>
<th>% Accuracy</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>128-176</td>
<td>152</td>
<td>141</td>
<td>131</td>
<td>138</td>
<td>137+-/-5</td>
<td>90</td>
<td>4%</td>
</tr>
<tr>
<td>Medium</td>
<td>453-575</td>
<td>514</td>
<td>461</td>
<td>450</td>
<td>461</td>
<td>457+-/-6</td>
<td>89</td>
<td>1%</td>
</tr>
<tr>
<td>High</td>
<td>962-1174</td>
<td>1038</td>
<td>960</td>
<td>984</td>
<td>1006</td>
<td>983+-/23</td>
<td>95</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 45: Intra-assay precision using Low, Medium, and High QC samples were analysed 3 times each in one run for intra-assay precision.

5.3.5 Patients' UAS7 Score Results.
See Chapter 3 for the score results and Chapter 6 for the result discussion.

5.3.6 Blood Tests Results for CRP, ESR, C3, C4, and Vitamin D
See Chapter 3 for the results and Chapter 6 for results discussion.

5.4 Statistical Results.

5.4.1 Analysis of IL-3 Levels in Saliva.
Analysis of IL-3 in the samples from healthy subjects found that 57% of the samples had levels below LOD of 7.4 pg/ml. However, greater percentages were below the LOD in all CU (58%), untreated CU (47%), and CU OMA treated (63%) groups Table 46. The number of values (n) below the LOD are indicated as a ratio of the number (N) of samples and percentage (%) of samples in the Table 46.

<table>
<thead>
<tr>
<th>IL-3 (LOD=7.4 pg/ml)</th>
<th>Healthy controls</th>
<th>All CU</th>
<th>CU untreated</th>
<th>CU OMA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;LOD (n/N)</td>
<td>17/30</td>
<td>25/43</td>
<td>7/15</td>
<td>17/27</td>
</tr>
<tr>
<td>&lt;LOD (%)</td>
<td>57</td>
<td>58</td>
<td>47</td>
<td>63</td>
</tr>
</tbody>
</table>

Table 46: Ratio and percentages < LOD in healthy controls, all CU, CU untreated and CU OMA treated

The number of values below the LOD for salivary IL-3 was high in all groups. A recent systematic review (Diesch et al., 2021) and further literature search indicated that there have been no previous reports of human salivary IL-3 concentrations, presumably because it is generally undetectable, as reported for mouse salivary IL-3 (Delaleu et al., 2008).
5.4.2 Fisher Exact Test IL-3 Data Analysis

In the first statistical analysis of the data, the Fisher Exact Test was applied to compare the proportions of samples from healthy controls and CU patients (untreated and treated with Omalizumab) with detectable or undetectable levels of IL-3 Table 47.

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>IL-3 negative</th>
<th>IL-3 positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>17</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>CU</td>
<td>25</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>31</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 47: IL-3 contingency table for all healthy samples and all CU samples

<table>
<thead>
<tr>
<th>Test</th>
<th>Fisher's exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant (P &lt; 0.05)?</td>
<td>No</td>
</tr>
<tr>
<td>Effect size</td>
<td>Value, 95% CI</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.4048, 0.2704 to 0.5551</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.5806, 0.4077 to 0.7358</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.5667, 0.3920 to 0.7262</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.4186, 0.2838 to 0.5667</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>0.9652</td>
</tr>
</tbody>
</table>

Table 48: Fisher’s exact test P-value and statistical significance for all healthy samples and all CU samples.

The data (Table 48) shows that there was no significant difference in the proportion of samples with detectable levels of IL-3 in healthy controls and CU patients.

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>Negative IL-3</th>
<th>positive IL-3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>17</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>CU untreated</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>21</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 49: IL-3 contingency table for all healthy samples and untreated CU samples
The data (Table 50) shows that there was no significant difference in the proportion of samples with detectable levels of IL-3 in healthy controls and untreated CU patients.

Table 50: Fisher’s exact test P-value and statistical significance for all healthy samples and untreated CU.

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>IL-3 negative</th>
<th>IL-3 positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>17</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td>OMA treated CU</td>
<td>17</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>23</td>
<td>57</td>
</tr>
</tbody>
</table>

Table 51: IL-3 contingency table for all healthy samples and OMA treated CU samples

Table 52: Fisher’s exact test P-value and statistical significance for all healthy samples and OMA treated CU samples.
The data (Table 52) shows that there was no significant difference in the proportion of samples with detectable levels of IL-3 in healthy controls and OMA-treated CU patients.

<table>
<thead>
<tr>
<th>Data analysed</th>
<th>IL-3 negative</th>
<th>IL-3 positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CU untreated</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>CU OMA treated</td>
<td>17</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>18</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 53: IL-3 contingency table for untreated and OMA-treated CU samples

<table>
<thead>
<tr>
<th>Test</th>
<th>Fisher's exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value</td>
<td>0.3465</td>
</tr>
<tr>
<td>P value summary</td>
<td>ns</td>
</tr>
<tr>
<td>One- or two-sided</td>
<td>Two-sided</td>
</tr>
<tr>
<td>Statistically significant (P &lt; 0.05)?</td>
<td>No</td>
</tr>
<tr>
<td>Effect size</td>
<td>Value</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>0.2917</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.5556</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
<td>0.4667</td>
</tr>
<tr>
<td>Negative Predictive Value</td>
<td>0.3704</td>
</tr>
<tr>
<td>Likelihood Ratio</td>
<td>0.6563</td>
</tr>
</tbody>
</table>

Table 54: Fisher’s exact test P- value and statistical significance for untreated and OMA-treated CU samples

The Fisher Exact Test indicates no significantly higher proportion of IL-3 positive samples between untreated and OMA-treated patients with CU (p=0.3465) Table 54.
5.4.3 Censored Salivary IL-3 Data Analysis

![Graph showing IL-3 levels for Control and CU groups]

Figure 30: Censored data for salivary IL-3 in samples from all healthy controls and all CU patients

The Mann-Whitney test comparing salivary IL-3 in all healthy control samples with all CU samples (OMA-treated or untreated) showed no significant difference ($p=0.96$) between groups (Values in pg/ml are given in Table 55).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Urticaria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>30</td>
<td>43</td>
</tr>
<tr>
<td>Minimum</td>
<td>5.230</td>
<td>5.230</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>5.230</td>
<td>5.230</td>
</tr>
<tr>
<td>Median</td>
<td>5.230</td>
<td>5.230</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>39.63</td>
<td>65.10</td>
</tr>
<tr>
<td>Maximum</td>
<td>187.4</td>
<td>748.9</td>
</tr>
</tbody>
</table>

Table 55: Values for salivary IL-3 (pg/ml) in healthy controls and all CU patients

The median (25th-75th percentile) value for salivary IL-3 in healthy controls was 5.23 pg/ml (5.23-39.63 pg/ml) and for patients with urticaria was 5.230 pg/ml (5.23-65.10 pg/ml) Table 55. IL-3 was not significantly higher ($p=0.8498$) by the Mann Whitney test in the saliva of patients with urticaria than normal control values.

Data from CU patient sample analysis was subsequently grouped according to Omalizumab treatment or not. Kruskal-Wallis ANOVA showed no significant difference
(p=0.8) in salivary IL-3 concentrations (pg/ml) between healthy control samples and untreated CU or OMA-treated CU, Table 56. Data is presented as individual values in Figure 3.0.

![Graph showing IL-3 concentrations in different groups](image)

Figure 3.1: Salivary IL-3 in CU untreated, omalizumab treated patients with urticaria and control healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>Healthy control</th>
<th>CU untreated</th>
<th>CU OMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of values</td>
<td>30</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td>Minimum</td>
<td>5.200</td>
<td>5.230</td>
<td>5.230</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>5.230</td>
<td>5.230</td>
<td>5.230</td>
</tr>
<tr>
<td>Median</td>
<td>5.230</td>
<td>7.900</td>
<td>5.230</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>39.63</td>
<td>66.70</td>
<td>53.90</td>
</tr>
<tr>
<td>Maximum</td>
<td>187.4</td>
<td>748.9</td>
<td>562.7</td>
</tr>
</tbody>
</table>

Table 5.6: Values for salivary IL-3 (pg/ml) in healthy controls, CU untreated and CU OMA patients
5.4.3.1 IL-3 ROC Analysis

Figure 32: ROC curve of salivary IL-3 for all patients (OMA-treated and untreated) and healthy controls

ROC analysis of the predictive value of salivary IL-3 to detect CU in any patient (OMA-treated or untreated) compared to healthy control patients was nonsignificant ($p=0.9598$), Table 57.

<table>
<thead>
<tr>
<th>Area</th>
<th>0.5035</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std. Error</td>
<td>0.06868</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.3689 to 0.6381</td>
</tr>
<tr>
<td>$P$ -value</td>
<td>0.9598</td>
</tr>
</tbody>
</table>

Table 57: Area under the ROC curve of salivary IL-3 for all patients (OMA-treated and untreated) and healthy controls
Figure 33: ROC of Salivary IL-3 data for untreated patients and healthy controls

ROC analysis of the predictive value of salivary IL-3 to detect untreated CU compared to healthy control patients was nonsignificant ($p=0.6131$), Table 58.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>0.5467</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.09166</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>0.3670 to 0.7263</td>
</tr>
<tr>
<td>P value</td>
<td>0.6131</td>
</tr>
</tbody>
</table>

Table 58: Area under the ROC curve of salivary IL-3 data for untreated CU patients and healthy controls
5.4.5 Sensitivity, Specificity, and Likelihood Ratio for IL-3

Table 59: Sensitivity, specificity, and likelihood ratio for IL-3

<table>
<thead>
<tr>
<th></th>
<th>IL-3</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sensitivity%</td>
<td>95% CI</td>
<td>Specificity%</td>
<td>95% CI</td>
<td>Likelihood ratio</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 2.100</td>
<td>66.67 to 71.11</td>
<td>53.33 to 61.14</td>
<td>69.77%</td>
<td>1.429</td>
</tr>
<tr>
<td>5</td>
<td>&gt; 4.650</td>
<td>53.33 to 70.61</td>
<td>53.33 to 69.77</td>
<td>1.286</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&gt; 5.150</td>
<td>53.33 to 70.61</td>
<td>53.33 to 69.77</td>
<td>1.143</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt; 6.550</td>
<td>53.33 to 70.61</td>
<td>56.67 to 69.77</td>
<td>1.231</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&gt; 8.650</td>
<td>56.67 to 69.77</td>
<td>56.67 to 69.77</td>
<td>1.077</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&gt; 10.55</td>
<td>56.67 to 69.77</td>
<td>56.67 to 69.77</td>
<td>0.9231</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&gt; 12.00</td>
<td>56.67 to 69.77</td>
<td>56.67 to 69.77</td>
<td>0.7692</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&gt; 12.45</td>
<td>56.67 to 69.77</td>
<td>60.42 to 75.41</td>
<td>0.8333</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>&gt; 14.70</td>
<td>63.33 to 65.51</td>
<td>78.13%</td>
<td>0.9091</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>&gt; 21.85</td>
<td>66.67 to 48.78</td>
<td>60.77%</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&gt; 28.85</td>
<td>70.52 to 83.34</td>
<td>1.111</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>&gt; 34.10</td>
<td>73.33 to 65.55</td>
<td>65.82%</td>
<td>1.25</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>&gt; 42.05</td>
<td>76.67 to 59.07</td>
<td>68.21%</td>
<td>1.429</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>&gt; 56.00</td>
<td>80.62 to 90.49</td>
<td>1.667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>&gt; 65.90</td>
<td>80.62 to 90.49</td>
<td>1.333</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>&gt; 70.10</td>
<td>80.62 to 90.49</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>&gt; 81.10</td>
<td>80.62 to 90.49</td>
<td>0.6667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>&gt; 95.35</td>
<td>83.33 to 66.44</td>
<td>92.66%</td>
<td>0.8</td>
<td></td>
</tr>
</tbody>
</table>

5.4.6 Negative Predictive Value and Positive Predictive Value of IL-3

There was no cut off value for IL-3 that gave the best combination of sensitivity and specificity chosen from the raw data, calculated using GraphPad prism, Table 59. The IL-3 ROC data was not significant when all data was analysed or only naive patients, so there are no NPV or PPV values. The above data suggests that salivary IL-3 cannot distinguish patients with chronic urticarial from normal.

5.4.7 Correlations

There were no significant correlations between salivary IL-3 and plasma biomarkers (ESR, CRP, C3, C4, VITD) in the patient group using non-parametric Spearman rank correlation testing.

5.5.0 Discussion

Interleukin-3 (IL-3) is a haematopoietic factor that belongs to the β common (βc /CD131) family of cytokines(Borriello et al., 2019; Hercus et al., 2018). It is known to be a pleiotropic growth factor that stimulates the proliferation, differentiation, and survival of pluripotent haematopoietic stem cells (Blalock et al., 1999; Farrar et al., 1989; Lindemann & Mertelsmann, 1995; Nishinakamura et al., 1996). Interleukin-3 has been known to
mediate emergency myelopoiesis but of late a growing body of literature has highlighted its critical role in adjusting innate immune effector functions in humans (Borriello et al., 2019). IL-3 engages the heterodimeric receptor CD123 which is composed of a shared βc subunit and a specific α subunit that signal through the JAK2/STAT5 pathway as well as other kinases (Hercus et al., 2018). More studies have uncovered the pivotal roles played by IL-3 in the development and progression of a range of diseases, namely multiple sclerosis, atherosclerosis, aortic dissection, allergic asthma, lupus nephritis, haematologic malignancies, sepsis, and rheumatoid arthritis (Borriello et al., 2019).

Importantly, the wealth of knowledge that has uncovered the involvement of IL-3 in chronic urticaria (Ferrer et al., 2003; Shimizu et al., 2008; Ye et al., 2014) makes IL-3 relevant to this study. It is now known that IL-3 is associated with exacerbated chronic urticaria symptoms (Ye et al., 2014).

An in vitro study by Ferrer et al. (2003) revealed that basophils primed with IL-3 released 15% more histamine than those tested without priming. Shimizu et al. (2008) also reported autocrine IL-3 upregulation of mast cells as instrumental in the exacerbation of chronic urticaria symptoms.

5.5.1 Summary of IL-3 Key Findings

The present study was carried out to investigate the potential usefulness of salivary IL-3 as a biomarker in chronic urticaria. IL-3 was measured by ELISA in the saliva of patients (n=43) and healthy controls (n=34) that were collected using a salivette. As far as it is known, this is the first study to compare total levels of IL-3 in the saliva of healthy subjects and chronic urticaria patients. Statistical analysis of these results showed no significant difference in IL-3 levels between the two groups although a few patients had high levels. IL-3 was not significantly higher by the Mann Whitney test in the saliva of patients with chronic urticaria than normal control values. This finding is not in line with previous studies which reported elevated IL-3 in the blood CU patients (Curto-Barredo et al., 2014; Ferrer et al., 2003; Shimizu et al., 2008; Ye et al., 2014; Zuberbier et al., 2018). Additionally, elevated IL-3 was also reported in skin lesions of CU patients (Hermes et al., 1999). It is difficult to compare these findings because different sample types and analytical methods were used. This study used saliva (ELISA) samples whereas the previous studies used blood (BAT) and skin lesions (Immunohistochemistry staining).
The ROC curve for salivary IL-3 was not significant ($p=0.9598$). The area under the curve ROC was 0.5035. The AUC suggests that salivary IL-3 cannot distinguish CU patients from normal. Further statistical analysis showed that there were no significant correlations between salivary IL-3 and blood biomarkers (ESR, CRP, C3, C4, VITD) in the patient group using non-parametric Spearman rank correlation testing. The slow recruitment of naïve patients coupled with research time constraints resulted in the study focusing on medicated CU patients. Most of the patients ($n=27$) in this study were on Omalizumab treatment and a few were naïve patients ($n=15$) with no record of treatment. It was not known for how long these patients had been on omalizumab.

The patients were further subdivided into naïve (untreated patients), omalizumab treated, and healthy control groups and analysed by the non-parametric, the one-way ANOVA (Kruskal-Wallis test, with Dunns multiple comparisons) There was no significant difference between any group for IL-3 values.

The ROC curve was also not significant for untreated CU vs healthy controls. IL-3 levels are suspected to be affected by the treatment of drugs used (Lippert et al., 2000). In this study, a variation of few low and high results was also observed in omalizumab treated patients and naïve patients. Omalizumab treatment is discussed in detail in Chapter 2 and Chapter 6.

On the contrary, a few studies have reported that IL-3 was undetectable in the plasma/serum of normal individuals although a few studies reported detectable IL-3 in healthy participants (Fu et al., 2016; Koike et al., 1995). Mangan et al (1993) detected IL-3 post bone marrow transplant but not in pre-transplant or in healthy donors. A few studies that have reported that IL-3 binds to heparin sulphate (Alvarez-Silva & Borojevic, 1996; Nguyen et al., 2019) and this could suggest the undetectable levels in biological fluids. Interestingly, in another study, IL-3Rα (IL-3 receptor) expression was undetectable on normal CD34⁺CD38⁻ hematopoietic stem cells but was overexpressed on CD34⁺CD38⁻ cells in patients with some myeloid and lymphoid conditions such as Fanconi Anaemia-acute myeloid leukaemia (FA-AML)(Du et al., 2011). Most of these previous IL-3 investigations were done in plasma/serum using ELISA or flow cytometry techniques.
5.5.2 Potential Impact of IL-3 on Clinical Practice

IL-3 has a very short half-life of 30 minutes at 37° and is slightly longer at 4°C (Garland et al., 1983). In this study, saliva samples were collected on ice and stored at -80°C. A cocktail of protease inhibitors was added to samples before they were frozen. Protease inhibitors preserve protein in saliva samples from imminent natural degradation by way of the digestive function of saliva proteases which are triggered during collection. This study revealed that saliva can be a sample of choice to analyse IL-3 using an ELISA technique. Six different healthy donor saliva samples and 5 diluent controls were spiked with 400pg/ml of recombinant IL-3, and accuracy and precision were assessed at 1:0, 1:2, 1:4, and 1:8 dilutions. The spike/recovery and the linearity of dilution assays for adoption in the quantification of cytokines in saliva samples showed a good %recovery of IL-3. The overall recovery expected was 100% if the addition of diluent and saliva did not influence IL-3 detection. High accurate results were obtained in terms of absolute quantity and exclusion of matrix effects for both calibrator diluent and donor saliva controls at 400 pg/ml, Table 39 and 40. Table 41 and Table 42 show high accuracy in the overall average % recovery at all concentration levels in both the calibrator diluent and donor saliva controls with an exception at 50pg/ml. The IL-3 test at 400pg/ml and 200pg/ml concentrations showed high precision, not exceeding a coefficient of variation of 10% in the calibrator diluent controls (Table 43). However, low precision was seen at 100pg/ml and 50pg/ml concentrations of calibrator diluent controls (Table 43).

The study further showed that the optimum signals for quantification could be obtained when saliva samples are diluted by 1:2 using the appropriate diluent. Accuracy for IL-3 by ELISA could be described as generally good when using the premade commercial QC material. It is important to note that the QC material used in this study was not designed for salivary samples but cell culture supernatants.

The quantity of albumin in samples and its capacity to bind or release larger proportions of ligands are believed to interfere with ELISA tests. Literature indicates that albumin, complement, lysozyme, fibrinogen, and paraprotein in samples interfere with ELISA by affecting the antigen-antibody binding ability (Tate & Ward, 2004). Regrettably, no total salivary albumin was measured in this study. It is therefore highly recommended to perform ELISA on multiple serially diluted samples first before embarking on saliva ELISA analysis. This first step helps to determine the optimum working dilutions for the analyte of interest.
5.5.3 Limitations

A limitation of this study is the small number of participants enrolled. The CLSI EP12-A2 ("EP12-A2 User protocol for evaluation of qualitative test performance; approved guideline," 2008) recommends that as a minimum testing should continue until results from at least 50 positive specimens are obtained and that at least 50 negative specimens using the comparative method should be obtained to determine the specificity of the candidate method. However, although this was the aim of this postgraduate study, it was not possible in the time frame and the study finally recruited 43 patients and 33 controls. The slow recruitment of naïve patients coupled with research time constraints resulted in the study focusing on medicated CU patients. There is a need for further work to be done on salivary IL-3 of a large sample size of naïve patients. Future research with a large sample size on CU naïve patients (CU patients who are not on treatment and whose symptoms are not controlled by medication) will help to confirm the results of this study.

5.5.4 Conclusion

As far as it can be ascertained there is no known work on salivary IL-3 levels in chronic urticaria. An important finding in this study was the successful recovery of IL-3 in human saliva. The precision and accuracy data characterise this ELISA as a suitable method for the quantification of IL-3 in human saliva.

Another important finding was that, although IL-3 was detected in the saliva of some participants, there was no statistically significant increase observed in the levels of IL-3 in the saliva of CU patients relative to healthy control subjects.

Hence, the salivary IL-3 results suggest that it is not a helpful biomarker in CU patients although plasma/serum IL-3 involvement in CU is documented in previous studies. Based on the results of this study, there is increasing evidence that salivary IL-3 results together with routine blood tests, ESR, CRP, FBC, C3C4, and Vitamin D are not helpful diagnostic markers in the assessment of CU patients.
Chapter 6: Overall Discussion

6.1 Summary of Key Findings
This study was carried out to investigate the potential usefulness of salivary sTREM-1, IL-8, and IL-3 as biomarkers of disease in patients with previously diagnosed chronic urticaria. The mean age of the CU patients was 42.6+-13 years; 70% were female and 30% were male. The present study indicates that women were affected twice as often as men, which agrees with previous reports in the literature (Gaig et al., 2004; Greaves, 2000). As far as it can be ascertained there are no previous reports in the literature regarding the relationships between disease and sTREM, IL-8, and IL-3 in the saliva of CU patients. This is the first study to compare total levels of sTREM-1, IL-8, and IL-3 in the saliva of healthy subjects and chronic urticaria patients. The salivary tests carried out were to determine if there was an association between salivary sTREM-1, IL-8, or IL-3 and CU. The presence of an association is potentially diagnostic for the disease.

6.1.1 Salivary Cytokines
In this study many of the results for salivary IL-3 and sTREM-1 lie below the level of detection (LOD) of the ELISA kits used for analysis. For IL-8, all measurements are higher than the lowest standard point (15.6 pg/ml) of the ELISA kit used. The LOD for the R&D Systems DuoSet ELISA to measure sTREM-1 according to the manufacturer’s instructions, has been reported as 23.2 pg/ml (Hasibeder et al., 2015) and for IL-3 measured using the R&D Systems Quantikine ELISA is reported to be 7.4 pg/ml (from the product insert). Hence, in the first statistical analysis of the data, the Fisher Exact Test was applied to compare the proportions of samples from healthy controls and CU patients (untreated and treated with Omalizumab) with detectable or undetectable levels of sTREM-1 and IL-3. Also, the data sets for sTREM-1 and IL-3 were censored and substituted with a constant value because some values were below the LOD. Therefore, in the data sets being analysed, the substituted value for IL-3 was 5.23 pg/ml. For sTREM-1 the substituted value was 16.41 pg/ml. These censored data sets were non-normally distributed and were analysed for differences between two groups using the Mann-Whitney test. Differences between healthy and untreated or OMA-treated groups were analysed using the Kruskal-Wallis ANOVA with Dunn’s multiple comparisons post-hoc test.

The Fisher Exact Test indicates a significantly higher proportion of sTREM-1 positive samples in patients with untreated CU compared to healthy controls ($p<0.0152$), and
between untreated and OMA-treated patients with CU \((p<0.049)\). The Fisher Exact Test indicates no significant higher proportion of sTREM-1 positive samples when all CU patients are compared to healthy controls \((p>0.2535)\). In the OMA- treated CU patients sTREM-1 is not significantly \((p>0.999)\) higher than healthy controls values.

The Mann-Whitney test comparing censored data of salivary sTREM-1 in all healthy control samples with all CU samples (OMA-treated or untreated) showed a significant difference \((p=0.0277)\) between groups, and values for sTREM-1 were significantly higher in the CU group. The Fischer’s Exact Test indicates no significant difference \((p>0.9999)\) observed in salivary IL-3 of healthy subjects and chronic urticaria patients. The Mann-Whitney test comparing censored data of salivary IL-3 in all healthy control samples with all CU samples (OMA-treated or untreated) showed no significant difference \((p>0.96)\) between groups. Significantly higher values were observed for the levels of IL-8 \((p<0.0001)\) in the saliva of CU patients relative to healthy control subjects.

The patients were further subdivided into naïve (untreated patients), omalizumab treated and healthy control groups, and salivary sTREM-1, IL-8, and IL-3 data analysed by non-parametric, unpaired, analysis. The one-way ANOVA (Kruskal-Wallis test, with Dunns multiple comparisons) showed that sTREM-1 was significantly \((p=0.0120)\) higher in naïve patients than healthy controls. Patients treated with omalizumab did not have sTREM-1 significantly higher than healthy controls values, although this could not be attributed to therapy because the study did not have matched/paired samples. However, the sTREM-1 median value was significantly higher in the naïve group than the healthy controls, indicating that the increase in salivary sTREM reflected some aspect of the pathogenesis of CU. There was no significant difference in the median values between healthy controls and omalizumab treated patients, indicating a potential response to omalizumab treatment. However, the numbers of patients in each group were relatively small, and further research analysing salivary sTREM-1 in a longitudinal study of patients before and after the initiation of omalizumab therapy is required to confirm these preliminary observations.

More interesting was the ROC curve analysis for sTREM-1 when the patients were subdivided into the above groups. The ROC curve for sTREM-1 in naïve (untreated) patients was significant \((p=0.0034)\) indicating the potential diagnostic value of salivary sTREM-1 in patients with CU. The ROC curve was just significant \((p=0.0402)\), indicating
a poor diagnostic value of CU when values from all groups were pooled and used in the analysis.

In the naïve (untreated) group, a salivary sTREM-1 value >27.15pg/ml gave 80.00% sensitivity (54.81% to 92.95%) CI, and 67.65% specificity (50.84% to 80.87%) CI and the likelihood ratio was 2.473. This higher sensitivity also shows the potential diagnostic value of salivary sTREM-1 in CU.

There is an overlap of results for untreated CU patients with the patients on omalizumab treatment. This may be due to the fact that patients with high sTREM-1 may have just started on omalizumab treatment and those with low sTREM-1 may have been on omalizumab treatment for some time. Also, there are a few extreme sTREM-1 values, whose influence on the significance was minimized by using the non-parametric tests. At this point, one should also take into consideration that proteinases produced by oral bacteria (lysine-gingipain produced by P gingivalis), or released from inflammatory leukocytes, particularly those associated with periodontitis, could well degrade sTREM-1 released in saliva (Bostanci, Thurnheer, et al., 2013). This would mean that the original concentration of sTREM-1 produced may be greater than that eventually detected in the salivary environment since the protease inhibitors was added at the point of storage. Sample collection, addition of protease inhibitors, and storage are described in detail in Chapter 2.

Salivary IL-8 was significantly higher in both groups of patients, naïve and omalizumab treated (p<0.0184), compared to healthy controls. There was no difference between the naïve and the omalizumab groups. Omalizumab appears to have no significant effect on IL-8 concentrations. The ROC curve for salivary IL-8 was again significant for untreated, (naïve) patients versus healthy controls (p<0.0108), indicating the potential diagnostic value for salivary IL-8 measurements in CU.

The fact that the omalizumab treated patients had low sTREM-, but not lower IL-8 compared to naïve patients suggests that the anti-IgE therapy is inhibiting an IgE dependent mechanism that generates sTREM-1 in CU. It is of much interest to find whether mucosal dendritic cells that express mTREM-1(Rudick et al., 2017) contribute to sTREM-1 in an IgE-dependent way.

Also, due to the strategic location of mast cells, they are among the first cells to interact with antigens, and thus they are key cells of immunological surveillance against infections.
Mast cells through mediators they release, recruit neutrophils, monocytes, the cells that release TREM-1 in the mucosa. If these mast cells are suppressed by omalizumab, they may not release these mediators, hence there is no recruitment of neutrophils and monocytes resulting in low sTREM-1. Even if there is an oral infection, it may not be enough to recruit enough cells to raise high sTREM-1. The balance between mast cells recruiting the neutrophils and monocytes versus local infection/inflammation recruiting the same cells suggests the mast cells may have a strong influence and that suggests the low sTREM-1 levels seen in this group.

In contrast, there was no significant difference between any group for salivary IL-3 values. The ROC curve for IL-3 was also not significant for naïve CU patients vs healthy controls. Furthermore, there were no significant correlations between salivary IL-8, TREM-1, or IL-3 and blood biomarkers (ESR, CRP, C3, C4, vitamin D) in the patient group. The lack of blood results from the healthy control group makes this comparison difficult to make.

Infection in the oral cavity of CU patients is one among many factors that can trigger CU. Hence, there is a need for the identification of biomarkers that are helpful in the diagnosis and prognoses of CU. Significantly higher levels of IL-8 and sTREM-1 in the saliva of CU patients relative to healthy control in this study suggest the persistent presence of bacterial infection in the oral cavity of CU patients. IL-8 and sTREM-1 may play a key role in the host response to infection in the oral cavities of CU patients, and the ROC analysis indicated the diagnostic potential of salivary sTREM-1 and IL-8 for CU patients.

This finding is directly in line with a previous finding that showed increased levels of sTREM-1 and IL-8 in an in vitro study that was testing the effect of oral bacterial on TREM-1 expression and production by the human polymorphonuclear cells (Bostanci, Thurnheer, et al., 2013). Although these findings agree, it is difficult to compare the findings because the studies are different. The present study tested salivary sTREM-1 and salivary IL-8 in CU patients whereas Bostanci, Thurnheer, et al (2013) used culture supernatants in an in vitro study. As it is highly suggested that oral infection flares up CU (Tadros et al., 2018), using salivary sTREM-1 and salivary IL-8 could be helpful to diagnose CU patients with oral infections as a cause of increased symptoms.

Previous studies that investigated sTREM-1 in periodontal diseases detected increased levels of this molecule in saliva and/or serum (Bostanci, Oztürk, et al., 2013; Nylund et al., 2018; Räisänen et al., 2020; Willi et al., 2014). A similar pattern of results was also
obtained in other studies that investigated salivary sTREM-1 in periodontal infections (Belibasakis et al., 2014; Bostanci & Belibasakis, 2012; Bostanci et al., 2011; Nylund et al., 2018; Räisänen et al., 2020; Willi et al., 2014). Bostanci et al (2013) investigated salivary and serum sTREM-1 levels in individuals without periodontitis and those with chronic or aggressive periodontitis. They observed higher salivary sTREM-1 concentrations in chronic and aggressive periodontitis than in the control group, by 3.3-fold and 5.6-fold, respectively.

Their values were higher (healthy controls, 212.18+32.23 pg/ml, chronic periodontitis, 914.45+170.99, aggressive periodontitis, 1127.16+13.63 pg/ml) than the values obtained in this study (healthy controls, 16.41 and CU patients, 32.0 pg/ml) even though they used mean values instead of median values. They also observed higher sTREM-1 concentrations in serum of chronic and aggressive periodontitis than in the control group, and the differences were 1.7-fold and 2-fold, respectively. Regrettably, the current study did not test serum sTREM-1. The sTREM-1 concentrations were two-fold, higher in saliva than in serum. These differences are likely to occur because oral sTREM-1 would be considerably diluted in serum compared with saliva.

The finding of high levels of IL-8 and sTREM-1 in this study is expected as it is suggested that TREM-1 mediates the amplification of cytokine production (Bostanci et al., 2011). It is also known that Porphyromonas gingivalis can instigate pro-inflammatory cytokine production by monocytes (Bostanci et al., 2007).

There is a convincing body of evidence that indicates elevated sTREM-1 is detected in fluids of patients suffering from bacterial infectious diseases (Gibot et al., 2005; Gibot, Cravoisy, et al., 2004; Kofoed et al., 2007; Liu et al., 2007), but that there are studies where serum/plasma sTREM-1 could not distinguish between infection and no infection. Increased sTREM-1 levels were detected in plasma in sterile inflammation for example in asthma where plasma sTREM-1 correlated with the neutrophil number (Bucova et al., 2012). Also, elevated sTREM-1 levels were detected in the gingival crevicular fluid (GCF) of elderly patients with gingivitis or periodontitis compared with elderly healthy controls (Öztürk et al., 2016). The increased concentrations in both the elderly healthy control and the elderly patient groups are suggested to be caused by a dysregulated immune response seen in elderly people. Rudick et al (2017) posit that the dysregulated immune response is due to compromised function of monocytes and macrophages e.g., reduced chemotaxis,
phagocytosis, production of reactive oxygen, and chemokine responses. Furthermore, the measurement of serum sTREM-1 concentrations in febrile neutropenic patients was not able to identify bacterial infections (Michel et al., 2017).

Studies that investigated IL-8 in oral lichen planus (Mozaffari et al., 2018) presented evidence that IL-8 is fully detected in saliva and that this salivary cytokine can be used as a diagnostic and therapeutic biomarker for oral lichen planus. Oral lichen planus is an autoimmune and chronic inflammatory condition that affects the oral mucus membrane (Drogoszewska et al., 2014) and may also involve the skin and the genitalia (Eisen et al., 2005). IL-8 has been identified to be an important molecule in the localisation, collection, and activation of neutrophils, and that it is induced and secreted by monocytes, lymphocytes, fibroblasts, and epithelial cells. It is also speculated that microorganisms such as Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, and Aggregatibacter actinomyctetemcomitans and the toxins they release in periodontal tissues stimulate the formation of IL-8 (Ertugrul et al., 2013; Mathur et al., 1996; Tsai et al., 1995). Other previous studies were broadly in line with having elevated GCF IL-8 in patients with periodontitis (Mathur et al., 1996; Tsai et al., 1995). However, some studies reported higher IL-8 levels in healthy control groups compared to patients with periodontitis. (Chung et al., 1997; Ozmeriç et al., 1998).

The findings in the current study suggest the capacity of sTREM-1 and IL-8 to be useful as diagnostic markers in CU patients. Although there was no statistically significant increase observed in the levels of IL-3 in the saliva of CU patients relative to healthy control subjects, an important finding in this study was the successful recovery of IL-3 in human saliva. The ELISA test was found to be a suitable method for the quantification of IL-3 in human saliva.

As far as it can be ascertained, very few clinicians take their time to examine the mouths and teeth of patients with chronic urticaria when they come for their clinical appointments. The results of this study suggest that all clinicians who see chronic urticaria patients in allergy clinics and other primary care clinics should perform oral examinations so that this tractable trigger, if present, for chronic urticaria is not missed. This finding is in line with a recent case study that treated oral infection of a CU patient leading to the resolution of CU (Tadros et al., 2018).
6.1.2 Blood Biomarkers

The diagnosis of CU has always been difficult and complicated. Current methods are clinically based. They involve history, physical examinations, provocation/challenge tests such as ASST and BHRA. Severity assessments using Urticaria-Activity-Score-7 (UAS7) are also applied. The most used diagnostic assays are the routine tests, C3, C4, Vitamin D, ESR, and CRP. The selection of a biomarker for CU, in general, has always been a challenge. This has resulted in a widespread of the unnecessary ordering of laboratory tests in the healthcare system leading to increased expenditure without the demonstrable benefit (Detsky & Verma, 2012). In this study, the levels of Vitamin D were within the normal reference range but on the lower end. The low levels of Vitamin D are in agreement with previous studies that have found Low vitamin D in CU patients than in healthy controls (Movahedi et al., 2015) and replacement therapy has been found to reduce the severity of the urticarial wheals (Oguz Topal et al., 2016; Rasool et al., 2015). Vitamin D beyond its role in bone physiology plays a central role in regulating inflammation and tissue damage in the skin. Its deficiency is suggested to result in excess inflammation that leads to degranulation of mast cells.

C3 and C4 were also normal in this study and are in line with a previous study that reported normal C3, C4 levels in CSU patients (Ghaffari et al., 2012). The measurement of C3 and C4 helps to differentiate urticaria vasculitis from chronic urticaria. Urticarial vasculitis patients have persistently low levels of C3 with normal C4 (Omoyinmi et al., 2018). C3 and C4 are components of the complement cascade. C3 has a central position in the complement cascade and it is the dominating complement factor in serum (Persson et al., 1999) and its function is to clear immune complexes and influencing antibody production (Tedder et al., 1994). C4 inhibits immune complex precipitation and induces haemolysis (Persson et al., 1999).

In this study, the levels of CRP and ESR were also within the normal reference range in both treated and naïve CU patients. CRP and ESR are the most common routine tests used to single out chronic urticaria at UHS. CRP and ESR are also commonly ordered in tandem to evaluate other patients with other conditions e.g. fever and inflammatory conditions (Kainth & Gigliotti, 2014). CRP is an acute phase protein produced by the liver and has been routinely used as a marker of bacterial infections. CRP has been found to differentiate between bacterial and non-bacterial infections (Ahn & Lee, 2012; de Jong et al., 2016; Hochreiter et al., 2009; Hochreiter et al., 2008; Qu et al., 2012; C. W. Wu et al., 2012).
2015). It is extremely sensitive and is currently considered to be the key biomarker of systemic inflammation or infection within the clinical practice (Aguiar et al., 2013; Pay & Shaw, 2019). It is mainly synthesised by hepatocytes in the liver and is regulated by cytokines, interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α) (Bennett & Plum, 1996; Corey-Bloom et al., 2020; Del Giudice & Gangestad, 2018; Ebersole & Cappelli, 2000).

CRP is usually present at relatively low levels in plasma/serum (below 10 mg/l), but it is known that bacterial infections cause a rapid elevation of CRP levels within hours of infection. Its levels rise up to 50,000-fold from baseline, doubling every 8 hours and peaking approximately 42 hours following an infection or tissue injury (Jain et al., 2011). The usefulness of these changes in plasma/serum CRP levels has been identified in the diagnosis of infections. The lowering of CRP levels is usually accompanied by the resolution of the disease. On the other hand, CRP is not useful in differentiating infectious inflammation from non-infectious inflammation. It is known that CRP is increased several folds after physical trauma and sustained levels of CRP elevation can accompany the active non-infectious inflammatory disease. Elevated CRP can also result from a pulmonary embolism, deep vein thrombosis, myocardial infarction, malignancies, rheumatoid arthritis, and autoimmune diseases. It is difficult to compare the CRP results of this study to previous studies. The CRP results in CU patients are in contrast with previous studies. CRP was within the normal range in this study and yet previous studies have noted increased levels of systemic CRP in patients with oral infections. Also, this study had no CRP results in the healthy controls. An early study detected increased levels of CRP in serum of patients with inflammatory oral disease- acute alveolar abscesses (Boucher et al., 1967). A recent review reported that systemic CRP is consistently elevated in people with oral infections (Hegde & Awan, 2019). Also, in a recent study, patients with severe periodontitis had increased serum levels of CRP (Gomes-Filho et al., 2011) but regrettably, their study could not indicate that periodontitis was the cause for the observed increased serum CRP levels. The increase in CRP levels could be due to other factors. CRP levels are known to increase in various confounding factors such as aging, high blood pressure, alcohol use, smoking, coffee consumption, diabetes, high protein diet, elevated triglycerides, sleeping disturbances and depression (Graziani et al., 2010).

Further studies have also shown a positive association between chronic periodontitis and high serum CRP levels (Ebersole et al., 1997; Loos et al., 2000; Noack et al., 2001; Slade
et al., 2003; Slade et al., 2000; Wu et al., 2000). Also, some studies have reported that periodontal treatment (oral hygiene, subgingival scaling, root planning, and tooth extraction) significantly lower serum CRP levels (D'Aiuto et al., 2004).

Unfortunately, the above studies did not compare the oral and systemic levels of CRP to confirm the source of the elevated CRP as some of the patients in these studies had cardiovascular diseases (Wu et al., 2000). Additionally, a multiple number of other previous studies have also assessed the correlation between salivary and serum CRP level in which a positive correlation has been detected, with the exception of a few studies that show no correlation (Bhavsar et al., 2015; Browne et al., 2013; Dillon et al., 2010; Foley et al., 2012; Gustafsson et al., 2011; Iyengar et al., 2014; Labat et al., 2013; Ouellet-Morin et al., 2011; Out et al., 2012; Punyadeera et al., 2011). Despite the inconsistencies, a moderate correlation between salivary and serum CRP has been established with a serum dilution factor of order $10^4$ (Bhavsar et al., 2015; Browne et al., 2013; Dillon et al., 2010; Foley et al., 2012; Gustafsson et al., 2011; Iyengar et al., 2014; Labat et al., 2013; Ouellet-Morin et al., 2011; Out et al., 2012; Phalane et al., 2013; Punyadeera et al., 2011).

Regrettably, these studies were investigating salivary CRP as a reflection of systemic inflammation. The elevated CRP could be as a result of the systemic disease since periodontal disease and systematic health have a two-way relationship (Hegde & Awan, 2019).

A separate study using oral fluid, the gingival crevicular fluid (GCF), detected increased levels of CRP (Kumar et al., 2013). GCF is a serum exudate that carries all major components of the blood including complement proteins, immunoglobulins, cells such as neutrophils and plasma cells, and the acute phase proteins (Megson et al., 2010; Taylor & Preshaw, 2016). Regrettably, this study did not compare the GCF CRP with serum CRP. There is limited use of GCF in research because its collection is considerably more difficult, and it requires specialist training and equipment.

Elevated levels of CRP have been consistently reported in CSU patients (Kolkhir et al., 2018) but the CSU patients in this study showed normal CRP levels. This association was not observed in the current study may be due to the small number of patients recruited, 43 compared to 1053 in Kolkhir et al (2018) study. Another suspected cause of normal serum CRP in CU patients could be due to the dilutional effect or that there was a mild or low level of oral infection that did not increase systemic CRP in circulation as indicated by the
measured levels of salivary sTREM-1 and IL-8 which are also markers of infection. Normal levels of serum CRP could also mean that the CU patients had no infections. Noteworthy, some studies have claimed that soluble TREM1 is a superior biomarker for predicting and diagnosing sepsis than CRP (Charles et al., 2016; Su et al., 2012).

CRP and other markers such as procalcitonin (PCT) have been found to differentiate between bacterial and non-bacterial infections (Ahn & Lee, 2012; de Jong et al., 2016; Hochreiter et al., 2009; Hochreiter et al., 2008; Qu et al., 2012; C. W. Wu et al., 2015). PCT is a protein biomarker for the presence and severity of bacterial infection whose level rises within 12 hours of bacterial involvement and decreases as the host immune system begins to control the infection (Peters et al., 2021). Unlike CRP and ESR, PCT levels remain low in non-bacterial causes of infection and inflammation (Wacker et al., 2013). However, some studies contrast the effectiveness of CRP and PCT. They have proven serum CRP and PCT to be ineffective and disappointing in some cases, with results showing that there is no difference in the levels of these proteins between patients with infection and those without (Akpinar et al., 2014; Brunkhorst et al., 2002; Duflo et al., 2002; Kim et al., 2017).

ESR was also normal in this study and is in line with a previous study that reported normal ESR levels in CSU patients (Ghaffari et al., 2012). This finding is in contrast with a recent study that detected increased ESR levels in CU (Akca & Tuncer Kara, 2020). The significantly higher ESR value in CSU patients could be due to the higher mean age of CSU patients in Akca & Tuncer Kara (2020) study since ESR increases with age (Miao, 2002). ESR is one of the first methods used to investigate pathological conditions. It is a non-specific method, and its levels are upregulated in many non-infectious conditions such as stroke and coronary artery disease. ESR is outperformed by CRP in terms of responsiveness and specificity for inflammation. CRP is not or less likely to be affected by size, shape, and number of red blood cells, female sex, and pregnancy as in ESR (Harrison, 2015). Also, false-negative, and false-positive results are less common for CRP than for ESR.

Of much concern is the continuous and unspecific use of CRP and ESR as biomarker tests for CU patients. Using these usual markers of infection is sometimes not helpful in CU
patients with gums and sinus bacterial infections as their levels would appear normal because infection of the gums and sinuses is localised. In addition, despite the BHRA and BAT being recognized as the gold standard for diagnosing some forms of CSU the assays are not fully standardised and are time consuming, sometimes only being used for research purposes (McGowan & Saini, 2013). Food challenge tests are time consuming and can be potentially harmful to the patients.

Bacterial culture is the gold standard method for detection and isolation of microorganisms in body fluids and airways of patients suspected of bacterial infections (Baker et al., 1996; Gibot, 2006, 2009; Gibot, Cravoisy, et al., 2004; Loftus et al., 2016; Rello et al., 2014; Wunderink, 1998). Regrettably, there were no culture tests done in this study. Depending on the diagnostic tool used in the detection of bacterial infection, there are delays in getting definitive quantitative bacterial culture results (Bauer & Reinhart, 2010; Brown et al., 2011; de Cueto, 2005). Culture requires more than 24 hours before a diagnosis can be made. During this waiting period, antibiotics are usually administered to patients and can be harmful to patients. The use of antibiotics can result in the development of multiresistant bacteria, with consequential hospitalisation and an increase in admission costs (Amyes, 1998; Birmingham et al., 1997; Wilke & Grube, 2010). Generally, the use of antibiotics gives a false sense of security whilst the patient’s condition is worsening as the real cause of fever is unknown (Bassi et al., 2014; Damas et al., 2015; Kalanuria et al., 2014; Lau et al., 2015). The need for a definite diagnosis is important regarding the choice of appropriate antibiotics and for hospital admission decisions (Akpinar et al., 2014; Brunkhorst et al., 2002; Duflo et al., 2002; Ehsanipour et al., 2017).

Noteworthy is the association of *Helicobacter pylori* with CU. Tan et al (2016) presented clear evidence of the association of CU with *Helicobacter pylori* infection but the current study did not investigate this association as *Helicobacter pylori* is a gram-negative bacteria usually found in the stomach. In the study by Tan et al (2016), a 21-35 kDa Mixed Protein Component from Helicobacter pylori was found to induce mast cell degranulation. The eradication of Helicobacter pylori is recommended as part of the routine chronic urticaria management as research has proven that *Helicobacter pylori* eradication demonstrated statistically significant benefits compared to untreated patients or *Helicobacter* negative controls without urticaria (Chiu et al., 2013; Wedi et al., 2009). However, these recommendations are still disputable (Goga et al., 1988).
It is also important to note the effects of stress on salivary markers of inflammation. CU patients have an increased level of stress because of the chronic nature of the disease. Salivary markers of inflammation were found to be increased in acute stress (Slavish et al., 2015). What is not known is whether these salivary markers are also increased in chronic stress since the patients in the present study have a chronic condition.

Most of the patients (n=27) in this study were on Omalizumab treatment and a few were naïve patients (n=15) with no record of treatment. It was not known for how long these patients had been on omalizumab. Omalizumab appears to have an effect on sTREM-1 and no effect on both IL-8 and IL-3 as discussed above.

The drug, omalizumab is a recombinant DNA-derived monoclonal antibody. It targets free IgE antibodies and downregulates FcεRI within 2-8 weeks on basophils and mast cells, respectively. There are risks of anaphylaxis therefore omalizumab is administered in a clinic/doctor’s office. However, the risk/benefits ratio makes it the drug of choice in the refractory CU (third-line treatment). Despite its success in treating refractory CU, cases of CU that are resistant to omalizumab exist (Viswanathan et al., 2013). The European guidelines, other international societies guidelines, and the British guidelines (Powell et al., 2015; Zuberbier et al., 2014), concur that chronic urticaria frustrates sufferers as well as clinicians due to the presence of varied triggers coupled with challenges in diagnosis hence the continued need for standardized approaches to treatment and robust diagnosis. When there is no clear trigger identified, patients tend to depend on medication as symptoms persist for years causing anxiety, depression, and reduced quality of life (QoL). Chronic urticaria sufferers have a reduced QoL that is similar to other life threatening ailments such as ischemic heart disease and preoperative coronary artery bypass grafting (Beck et al., 2017).

6.2 Potential Impact on Clinical Practise
The determination of raised salivary IL-8 and salivary sTREM-1 in CU patients in this study is novel and ROC analysis suggests that alone or in combination, these cytokines may be diagnostic of CU.

Any patient with elevated salivary IL-8 (suggested cut-off point; >168.5 pg/ml) and salivary sTREM-1 (suggested cut-off point > 27.15 pg/ml), would be referred to the dentist and/or to the ear, nose, and throat (ENT) clinic for further clinical investigations and treatment.
Saliva has been used widely in disease monitoring. The potential benefits of saliva have resulted in increased research leading to some established clinical uses, for example in assessing cortisol, immunoglobulin A, and screening for immunodeficiencies (Castagnola et al., 2017; Nunes et al., 2015). There are major obstacles that need to be overcome when using saliva and these include the influence of the oral environment on the investigated biomarker, collecting a standardised sample, and extracting the biomarkers reproducibly from the complex saliva matrix (Granger et al., 2012; Nunes et al., 2015). However, there are several potential advantages of using salivary markers in this study.

Saliva analysis has some obvious advantages compared with blood-based analyses such as easy, safe access, and non-invasive collection. The method of collection does not require specialist training or equipment and places, and the collector is at reduced infection risk compared to blood collection. In addition, saliva lends itself to repeated sampling in longitudinal studies and collection from difficult patients in non-clinical settings.

### 6.3 Future Work:

Understanding the full effects of sTREM-1, IL-8, and IL-3 in chronic urticaria patients is an issue for future work. The current study had some limitations mainly related to the sample size. Future research should further develop and confirm the initial findings in studies using a larger patient sample size. Apart from investigating salivary sTREM-1 and IL-8, future work should also investigate serum sTREM-1 and IL-8 levels in a large sample size. If the serum levels of these biomarkers are low this could reflect local/focal infections in the oral cavity.

IL-3 levels were not statistically significantly different in CU in this study, regardless, further research is required to explore the levels in a large sample size which may become significant with larger cohort size. Future studies could also fruitfully explore these findings further by investigating the levels of the infection and inflammatory biomarkers such as salivary and plasma CRP and PCT.

No bacterial cultures were done in the current study. Bacterial cultures may constitute the object of future research of this study. Bacterial culture is the gold standard method for the detection and isolation of microorganisms in body fluids and airways of patients suspected of bacterial infections. Future investigations should also include patient oral inspections as they are necessary to validate the conclusion drawn from this study. There is a great need to record dental scores on all new patients during the next study. Oral inspections
accompanied by a cavity scoring system will help to determine whether the recruited CU patients have periodontitis. In addition, a prospective longitudinal study might prove an important study for future research. This should include measuring the levels of basophils, neutrophils, total IgE, cytokines (IL-8 and IL-6), CRP, and sTREM-1 in naïve CU patients on recruitment and then follow them up over time. There is also a need to follow up on the levels of IL-8 and sTREM over time from the day the patients start on omalizumab and other treatments.

6.4 Conclusion
As far as it can be ascertained, this is the first study to report salivary sTREM-1, IL-8, and IL-3 in chronic urticaria patients. The data from this study showed raised salivary sTREM-1 and IL-8 in chronic urticarial patients compared to normal controls. These elevated levels of salivary sTREM-1 and IL-8 suggest the persistent presence of oral bacterial infection in CU patients, although no infection analysis was done in the patients in this study. sTREM-1 and IL-8 may play a key role in the host response to infection in the oral cavities of CU patients. Based on these results, the main conclusion that can be drawn from this study is that these findings provide the potential use of sTREM-1 and IL-8 in the diagnosis of chronic urticaria patients with oral infection. The data also showed that there was no significant difference in salivary IL-3 of healthy subjects and chronic urticaria patients. The results cast a new light on biomarkers that may be added to a list of other biomarkers that are already used to aid the diagnosis of CU. Using salivary sTREM-1 and IL-8 together with the other biomarkers may therefore help to accelerate the diagnosis and treatment of chronic urticarial patients.
Chapter 7. Reflection

7.0 My Journey on the DBMS and the Project
Reflecting on my journey as a Doctorate of Biomedical Sciences (DBMS) student allows me to recognise areas I need to improve to become a successful learner. The DBMS course attracts professionals who like me, are in senior roles mostly in healthcare settings. My journey on the DBMS course commenced with a taught unit that comprised of the following modules: Professional Review and Development, Advanced Research Techniques, Publication and Dissemination, and Project Proposal. Lecturers with expertise in their respective subject areas delivered lectures, seminars, and tutorials to us. Interactive workshops and group discussions, as well as PowerPoint presentations, were included to facilitate learning. On completion of each module, we had to submit an assignment that was used to assess our understanding of the coursework/module.

The modules I completed in the taught unit enabled me to redefine myself as a higher learning student. Furthermore, I was motivated and challenged to work hard on each module to ensure I would be able to submit the assignments timeously. I felt supported on this unit and I was able to request assistance with the coursework when I required help. Fundamentally (most importantly), the taught Unit allowed me to scrutinise the gaps in my knowledge (my weaknesses) and reflect on ways to overcome these challenges to improve my learning outcomes. In summary, following this unit, my confidence in public speaking and conducting scientific discussions has greatly improved. In addition to the taught unit, I also attended many graduate school development programmes (GSDPs). The various courses in GSDP enabled me to develop ways to challenge my weaknesses resulting in improved skills and knowledge. Following GSDP, I gained confidence in presentations, Advanced MS Word, SPSS, writing a thesis, and publication of articles. Moreover, I have used the skills I gained to improve my practice on various platforms such as at work and supporting students as a lecturer at the university level.

7.1 Professional Review and Development Module
This module was very essential in my journey on the professional doctorate programme as it provided me with the opportunity to review my strengths and weaknesses objectively. I was able to identify my weakness in time management and plan ways to improve and overcome this weakness. Additionally, the Professional Review and Development module
helped me to set goals for the rest of the programme and these goals are reviewed regularly by myself and my supervisor and also annually during my appraisal meeting.

7.2 Advanced Statistics and Research Methodology
This module covered both quantitative and qualitative statistical methods. I have chosen quantitative methodology for my study because my research study is quantitative. I particularly enjoyed this module as I love working with numbers. The impressive results I achieved on the assignment reflect how much I enjoyed this module. Although my study area is mainly quantitative, learning qualitative research methods has broadened my knowledge and understanding of how other studies are carried out. Learning the two research methods has equipped me with the knowledge and confidence to supervise students who will choose either quantitative or qualitative research methods in the future. Furthermore, this module has taught me the flexibility and understanding of using different statistical software.

While we were taught how to use Statistical Package for the Social sciences (SPSS) on this module, I opted to use GraphPad Prism for the statistical analysis for my study as I found it easier. Knowledge of various statistical processes through the DBMS programme has enabled me to present data visually even to lay people. I have used the skills I gained on this module during presentations I have carried out across various platforms such as Zoom meetings. On one of the Zoom meetings I held, I had an audience of over 200 participants/people and my knowledge of the statistical presentation of data enabled the audience to understand the numbers easily.

7.3 Publication and Dissemination
I had an incredible opportunity to collaborate with other scientists resulting in the publication of two journal articles during this programme. The articles were published in OMICS: A Journal of Integrative Biology. The titles of the articles are 1. Coronavirus Disease-2019 Treatment Strategies Targeting Interleukin-6 Signalling and Herbal Medicine. 2. Integrating Artificial and Human Intelligence: A Partnership for Responsible Innovation in Biomedical Engineering and Medicine.
These articles are not related to this thesis, however, I have plans to publish an article from this thesis on completion.

### 7.4 Presentations

I had a chance to present a poster of the initial findings of my study to assessors and fellow students on annual review days. The feedback I received from assessors and fellow students has been positive and this has significantly helped improve my presentation skills. I am applying the presentation skills I have acquired in delivering lectures to students and also in meetings. During the pandemic, I was elected as the COVID 19 National Task Force Chairman by my United Kingdom (UK) church organisation and my responsibilities include training, teaching, and informing church members about COVID 19 virus and the available vaccines. This responsibility has been challenging as 95% of my church members are ethnic minorities, like myself. It has been reported that ethnic minorities have been disproportionately affected by the pandemic, moreover, the uptake of the vaccine by ethnic minorities has been poor. I have found that the PowerPoint presentation skills I gained from the DBMS programme have been an extremely useful tool when I deliver teachings about the virus and vaccination to my church members on virtual platforms such as Zoom.

### 7.5 Annual Reviews

The annual reviews present an opportunity for my assessors to evaluate my progress and offer constructive criticism and guidance on my work. Fellow students on the same course also provide feedback and comments that are vital to my work. Likewise, I have had the chance to review my fellow students’ work, and this has assisted in assessing my progress compared to that of my fellow students. The interaction with professional colleagues...
during annual review meetings has helped increase my confidence. These review meetings have been essential in helping me keep on track with my research.

7.6 Networking
The DBMS programme helped to extend my academic tentacles as the programme gave me the confidence to approach my former lecturers at the University of Zimbabwe to volunteer my service to them. I was allowed to teach first-year Biomedical Science students on one of my visits to Zimbabwe. This rewarding experience helped build my confidence as a public speaker/lecturer. I was further motivated to continue offering my service as a lecturer by the positive feedback I received from students. I have since applied for the position of Adjunct Lecturer for the University of Zimbabwe.

The University of Zimbabwe Department of Laboratory Diagnostic and Investigative Sciences initiated the programme of improving medical laboratories in Africa in collaboration with the Cape Peninsula University of Technology Department of Biomedical Sciences-South Africa. They also work with Stellenbosch University Division of Chemical Pathology – South Africa and Africa Federation of Clinical chemistry and Laboratory Medicine and Empowered School of Health. These universities hold several workshops and discussions to improve diagnostic services in Africa. I have been invited to the workshops and I contributed positively to the discussions.

7.7 Supervising University Students and Training Laboratory Staff at UHS
This Professional Doctorate has equipped me with the knowledge and expertise to train other members of staff (new and old) in the department. I have managed to support 3 students with their MSc projects. One of the students and I were invited to Finland, Helsinki in 2015 to present our findings of IL-3 studies at the European Academy of Allergy and Clinical Immunology (EAACI) conference. We presented a poster on the Validation of a salivary Interleukin-3 Immunosorbent assay for the evaluation of Chronic urticaria exacerbated by a salicylate-rich diet.

7.8 Constraints
I was planning to complete the DBMS programme in 2018, however, I did not manage to complete it due to a critical health problem that occurred on 23rd September 2017. On the fateful Saturday morning, 3 days after completing my laboratory experiments, I woke up with a severe, excruciating headache, nausea, and vomiting and I could not open my eyes.
I was taken to the Emergency Department where a CT scan revealed that I had an aneurysmal subarachnoid haemorrhage. I was immediately taken to theatre where the aneurysm was clipped under general anaesthetic by a consultant neurosurgeon. Following this emergency brain surgery, I had persistent left-sided headaches for two months. The headaches progressively got worse until I was readmitted to the hospital on 29th November 2017 where another CT scan showed I had a subdural haemorrhage. A Burrhole for parietal chronic subdural hematoma (CSDH) was performed on 30th November under general anaesthesia. Following the second surgery, I had constant headaches and insomnia. My neurosurgeon informed me these were some of the post-operative side effects and I would potentially suffer from these side effects for a long time. I was strongly advised to avoid any pressure or stressful situations as this would help reduce the post-operative complications. I took 6 months of sickness absence from work, however, the headaches persisted until October 2019. This left me with no choice but to suspend my studies for 2 years. I tentatively re-started my studies in January 2020, however, on several occasions, the excruciating headaches would stop me from reading or writing my thesis. This was further compounded by the pressures of being a full-time employee when I returned to work as I struggled to fit in my thesis writing while experiencing ongoing health issues. I was advised to withdraw from the programme on several occasions by concerned friends as they feared my health issues would relapse. There were times when I also doubted my ability to continue the programme, however, the support I received from my family, supervisors, and colleagues has made it possible for me to get this far and submit my thesis despite the serious health challenges I faced. I am truly thankful to God and the neurology team who saved my life and helped restore my health.

7.9 Volunteering on the Ebola Frontline

When the largest and most complex Ebola outbreak (2015) recorded in modern times occurred in West Africa, University of Portsmouth students were among the first to offer their support. I was fortunate enough to be one of the four students on the Professional Doctorate in Biomedical Science course who volunteered to take their specialist laboratory clinical and training skills to Sierra Leone, the country hardest hit by the outbreak. I spent approximately six weeks in Sierra Leone. The rising cases of the Ebola virus and deaths motivated me to volunteer as I wanted to help people who were suffering.
Although going to Sierra Leone was not an easy decision to make as it meant I would be leaving my family in the UK for a long time, volunteering gave me “heart-humbling job satisfaction”. In the future, I will not hesitate to volunteer again if another crisis arose that needed my expertise as my experience in Sierra Leone was very rewarding. I worked in the Ebola treatment centre in Kerrytown, which was open 16 hours a day with three shifts analysing blood or mouth swab samples from suspected Ebola patients from across the western region of Sierra Leone. The experience I gained from the Ebola pandemic has been put to good use in the current COVID 19 pandemic. When this outbreak started the UHS pathology department management asked me to support the department responsible for testing swab samples using the PCR method in the Molecular Virology department and this is what I have been doing since March 2020. The lab is very busy as we test thousands of samples per day, and it has been very difficult to balance my study time with a very busy work environment. The department kindly allowed me to have one study day per week, however, this has not been enough, and I have had to use my annual leave days to ensure my thesis is completed.
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Rignault-Bricard, R., Machavoine, F., Mecheri, S., Hermine, O., Schneider, E., Dy, M., & Leite-de-Moraes, M. (2018). IL-3-producing basophils are required to exacerbate airway hyperresponsiveness in a murine inflammatory model. *Allergy, 73*(12), 2342-2351. [https://doi.org/10.1111/all.13480](https://doi.org/10.1111/all.13480)


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Appendices

Appendix A: Study Information Sheet

University Hospital Southampton

NHS Foundation Trust
Department of Immunology
C Level, South Block
Southampton General Hospital
Tremona Road
SO16 6YD
Tel. 02381206650

Information Sheet

Patients & Controls
Access to saliva samples for research purposes

LREC number: 14/NI/1089

1) Study Title:
Evaluation of Triggering Receptor Expressed on Myeloid Cells- 1 (TREM-1) as a marker of infection.
Short Title: Evaluation of Triggering Receptor Expressed on Myeloid cells -1

2) Introduction
You are being invited to take part in a research study. This study is being undertaken as part of an educational qualification. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. This study has been given a favourable opinion by HSC Research Ethics Committee A.

3) What is the purpose of this study?
Chronic infections are an important cause for urticaria but in many cases there is no clear source of infection. If the infection can be identified and treated, this will help treat the urticaria. In this study, we are trying to discover whether bacterial infection in our gums and sinuses could be a contributing factor for the urticaria. We aim to do this by measuring a protein in saliva called Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1).

TREM-1 is raised in patients with bacterial infections and it is thought to be a better test (more sensitive and specific) than the ones currently used

In this study we will be using saliva as a sample of choice. We will then compare levels TREM-1 in urticaria patients and individuals who do not have this disease.

4) Why have I been chosen?
We are asking both patients with urticaria and individuals without urticaria to volunteer to take part in this study. We would like to compare our findings in urticaria patients to results from those without this disease and this is the reason for approaching you.
5) Do I have to take part?
It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. If you decide not to take part, this will not affect the standard of care you receive.

6) What will happen to me if I take part?
During your appointment, your doctor will arrange for a saliva sample to be collected once and you will not be required to submit more samples in the future.

We would like to be able to study your saliva sample by various methods, which may require long-term storage (freezing).

7) What are the side effects of taking part?
There are no side effects involved in taking part. An ordinary saliva sample will be taken and tested in the lab for this study.

8) What are the possible benefits of taking part?
There will be no direct benefit to you from taking part in this study. The information we get from this study may help us to understand better the association of urticaria and bacterial infections of the gums and sinuses.

9) Will my taking part in this study be kept confidential?
All information collected about you will be kept strictly confidential. Any results from our laboratory tests will have your name removed so that you cannot be recognised from them.

10) What will happen to the results of the research?
The results will hopefully be published but you will not be identified in any report or publication.

In the extremely unlikely event, we happen to identify anything significant from your saliva sample during the course of this study, you will be informed by your doctor during a clinic appointment.

11) Contact for further information:
Dr Efrem Eren on 02381206650 or Mr Witness Dzobo on 02381204620 for details specific to this study.
Thank you for taking the time to read this leaflet
CONSENT FORM – Control Group

Title of Project:
Evaluation of Triggering Receptor Expressed on Myeloid Cells- 1 (TREM-1) as a marker of infection.

Short Title: Evaluation of Triggering Receptor Expressed on Myeloid cells -1.

LREC number:

Name of Researcher:

Name of Patient ________________________________

Please initial box

1. I confirm that I have read and understand the information sheet (Version 1 dated 21/01/2014) for the above study and have had the opportunity to ask questions

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by researchers from the Department of Immunology involved in this study, as well as by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research.
I give permission for these individuals to have access to my records.

4. I agree to take part in the above study.

___________________  ___________________  ___________________
Name of Patient       Date                  Signature

Dr E Eren & Mr W Dzobo

___________________  ___________________  ___________________
Researchers          Date                  Signatures

1 for patient, 1 for researcher, 1 to be kept with hospital notes
Appendix C: Consent form for Patient Group

University Hospital Southampton
NHS Foundation Trust
Department of Immunology
C Level, South Block
Southampton General Hospital
Tremona Road
SO16 6YD
Tel. 02380 796650

CONSENT FORM-Patient Group

Title of Project:
Evaluation of Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1) as a marker of infection

Short Title: Evaluation of Triggering Receptor Expressed on Myeloid Cells -1

LREC number:

Name of Researcher: Dr. E. Eren

Name of Patient ________________________________

Please initial box

1. I confirm that I have read and understand the information sheet (Version 1 dated 21/01/2014) for the above study and have had the opportunity to ask questions

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my
medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from the Department of Immunology at Southampton General Hospital, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study.

___________________  ___________________  ___________________
Name of Patient         Date               Signature

Dr E Eren & Mr W Dzobo

___________________  ___________________  ___________________
Researchers           Date               Signatures

1 for patient, 1 for researcher, 1 to be kept with hospital notes
Appendix D. Research Ethics Committee Certificate

Office for Research Ethics Committees
Northern Ireland (ORECNI)

Customer Care & Performance Directorate
Lissue Industrial Estate West
Rathdown Walk
Moira Road
Lisburn
BT28 2RF
Tel: 028 95361400
www.orecni.hscni.net

HSC REC A

23 February 2016

Mr Witness Dzobo
Biomedical Scientist 2
University Hospital Southampton
Haematology & Transfusion Department
Level D, Tremona Road.
Southampton
SO16 6YD

Dear Mr Dzobo

Study title: Evaluation of Triggering Receptor Expressed on Myeloid Cells- 1 (TREM-1) as a marker of infection.

REC reference: 14/NI/1089

Amendment number: Minor Amendment #1

Amendment date: 11 February 2016

IRAS project ID: 105842

Thank you for your letter of 11 February 2016, notifying the Committee of the above amendment.

The Committee does not consider this to be a “substantial amendment “as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not
therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received.

The documents received were as follows:

<table>
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<th>Document</th>
<th>Version</th>
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<tr>
<td>Notice of Minor Amendment (Additional tests)</td>
<td>11 February 2016</td>
</tr>
<tr>
<td>Other [Sponsor Confirmation - Minor Amendment]</td>
<td>15 February 2016</td>
</tr>
<tr>
<td>Research protocol or project proposal</td>
<td>Received 22 February 2016</td>
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Providing Support to Health and Social Care

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

14/NI/1089: Please quote this number on all correspondence

Yours sincerely

Katrina Greer
HSC PRS Manager

Email: prs@hscni.net
# FORM UPR16

## Research Ethics Review Checklist

Please include this completed form as an appendix to your thesis (see the Research Degrees Operational Handbook for more information).

### Postgraduate Research Student (PGRS) Information

<table>
<thead>
<tr>
<th>Student ID:</th>
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<tr>
<td>PGRS Name:</td>
<td>Witness Dzobo</td>
</tr>
<tr>
<td>Department:</td>
<td>School of Health Sciences and Social Work- Professional Doctorate in Biomedical Sciences</td>
</tr>
<tr>
<td>First Supervisor:</td>
<td>Professor Janis Shute</td>
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### Start Date: (or progression date for Prof Doc students)

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<td>Professional Doctorate</td>
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### Title of Thesis:

| EVALUATION OF SALIVARY SOLUBLE TREM-1, IL-8, AND IL-3 AS DIAGNOSTIC MARKERS IN CHRONIC URTICARIA AT UNIVERSITY HOSPITAL SOUTHAMPTON |

### Thesis Word Count:

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(excluding ancillary data)

If you are unsure about any of the following, please contact the local representative on your Faculty Ethics Committee for advice. Please note that it is your responsibility to follow the University’s Ethics Policy and any relevant University, academic or professional guidelines in the conduct of your study.

Although the Ethics Committee may have given your study a favourable opinion, the final responsibility for the ethical conduct of this work lies with the researcher(s).

### UKRIO Finished Research Checklist:

(If you would like to know more about the checklist, please see your Faculty or Departmental Ethics Committee rep or see the online version of the full checklist at: [http://www.ukrio.org/what-we-do/code-of-practice-for-research/](http://www.ukrio.org/what-we-do/code-of-practice-for-research/))

<p>| a) Have all of your research and findings been reported accurately, honestly and within a reasonable time frame? | YES ✓ NO |
| b) Have all contributions to knowledge been acknowledged? | YES ✓ NO |
| c) Have you complied with all agreements relating to intellectual property, publication and authorship? | YES ✓ NO |</p>
<table>
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**Candidate Statement:**

I have considered the ethical dimensions of the above named research project, and have successfully obtained the necessary ethical approval(s)

<table>
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If you have *not* submitted your work for ethical review, and/or you have answered ‘No’ to one or more of questions a) to e), please explain below why this is so:

Signed (PGRS): [Signature]

Date: 05/07/2021