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Immunohistochemical characterisation of inflammatory, neuroimmune and histologically "normal" appendicitis

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Doctor of Philosophy

By

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Declaration

The research presented in this thesis is bona fide original work carried out by the author: Mr Emmanouil Psaltis, PhD student, at the University of Nottingham between November 2013 and July 2018. All work was performed by the author, with the technical support of Mr Christopher Nolan and Mrs Maria Diez Rodriguez. Additional statistical advice was obtained from Professor Keith R Neal.

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Contents

Abstract

Background

Appendicitis is the most common surgical condition, but its diagnosis can be challenging. The practice of early appendicectomy to minimise morbidity and mortality has resulted in an increase of histologically "normal" appendices (HNA) being resected. Even, in the laparoscopic era, the removal of a macroscopically normal appendix in a symptomatic patient remains a clinical dilemma. The discrepancy between the clinical and histological diagnosis could suggest that an unknown pathology exists. This study compared the *in-situ* expression of key inflammatory markers in HNA resected from symptomatic patients with histologically inflamed samples.

Methods

The study retrospectively included 448 appendix specimens allocated in 4 groups. Group I (n=120) included specimens with histologically confirmed uncomplicated appendicitis, Group II (n=118) included samples with histologically confirmed complicated appendicitis (gangrene/perforation), and Group III (n=104) included appendices with no evidence of inflammation on conventional histology (HNA). The control group (n=106) consisted of appendices removed as part of the resected colon following elective colectomy. The expressions of TNF-α, IL-6, IL-2R and serotonin were studied with immunohistochemistry. Clinical data on signs and symptoms, routine laboratory tests and intraoperative findings were collected from the patient notes. Statistical analysis was carried out in SPSS version 21.

Results

TNF-α expression in HNA was significantly increased compared with the control and inflamed appendices ($p<0.05$). HNA also demonstrated significantly increased IL-6 expression in the epithelial cells compared with the control as well as inflamed samples ($p<0.05$). The IL-2R expression of the HNA was significantly increased compared with the control appendices ($p<0.05$) but not as high as in the inflamed samples ($p<0.05$). HNA did not demonstrate significantly different serotonin contents of enterochromaffin cells compared with the control group ($p=0.60$). The inflamed samples were significantly depleted ($p<0.05$). Patients with HNA demonstrated significantly elevated white cell count compared with the control group ($p<0.05$) but not as high as in patients with inflamed samples ($p<0.05$). Of patients with HNA 23% reported previous episodes of RIF pain compared with 14% and 4% of patients with uncomplicated and complicated appendicitis respectively, whereas the severity of RIF pain did not vary between the three groups of patients. Localised peritonism was present in 56% of patients with HNA compared with 73% of patients with inflamed samples. The neutrophil to lymphocyte ratio and C-reactive protein concentrations of patients with HNA were significantly lower compared with the control group as well as patients with inflamed appendices ($p<0.05$). Faecoliths were found in 27% of HNA compared with 11% and 28% of uncomplicated and complicated appendicitis samples respectively. Intraoperatively, 51% of the HNA were thought to be inflamed. The expression of the studied markers between samples with and without faecolith and between those that appeared normal and those that appeared inflamed was not statistically significant.

Conclusion

It was demonstrated that histologically "normal" appendices resected following a clinical diagnosis of acute appendicitis exhibited increased levels of TNF-α, IL-6 and IL-2R that could indicate an active inflammatory response. Signs and symptoms as well as routine laboratory results that help clinicians in the diagnosis of appendicitis were also abnormal in patients with HNA. These observations could either represent an inflammatory response at such an early stage that could not be detected with conventional microscopy or could indicate an abnormally long inflammatory response with features of chronicity. In addition to this, the fundamental differences between samples with complicated and uncomplicated appendicitis could suggest that the different degrees of inflammation in the appendicitis spectrum could be due to differences in the underlying immunopathogenesis and not just disease progression.

Presentations

Presentations to national/international conferences originated from this study:

"Histologically normal appendices are not always normal".

Psaltis E, Zaitoun AM, Lobo DN.

Oral presentation: 13/08/2017, World Congress of Surgery, Basel, Switzerland

"Are histologically normal appendices normal?"

Psaltis E, Zaitoun AM, Lobo DN.

Oral presentation: 06/01/2016, SARS, Royal College of Surgeons of England, London, UK

"Immunohistochemical characterisation of histologically normal and inflamed appendices in patients with right iliac fossa pain".

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Abbreviations

ACTH adrenocorticotropic hormone

APC antigen presenting cells

ARP actin related proteins

CCK cholecystokinin

CD Crohn's disease

CGRP calcitonin gene related peptide

COX cyclooxygenase

CRP C-reactive protein

CTL cytotoxic T lymphocyte

DAB diaminobenzidine

DARE deletion of AU-rich elements

DAMP damage-associated molecular pattern

DC dendritic cells

ECC enterochromaffin cell

ENS enteric nerve system

Fab antigen-binding fragments

FAE follicle associated epithelium

Fc crystalline fragment

GABA gamma-aminobutyric acid

G-17 gastrin

GALT gut-associated lymphoid tissue

GAP growth associated protein

GAPDH glyceraldehyde 3-phosphate dehydrogenase

GFA glial fibrillary acidic protein

GFAP glial fibrillary acidic protein

GI gastrointestinal

GP general practitioner

H&E haematoxylin and eosin

HMGB human group box protein

HRP horseradish peroxidase

HSP heat shock protein

HSP heat shock protein

IBD inflammatory bowel disease

IBS irritable bowel syndrome

IFN interferon

Ig immunoglobulin

IHC immunohistochemistry

ICC immunocytochemistry

IL interleukin

ILF isolated lymphoid follicle

IQ interquartile

MALT mucosal-associated lymphoid tissue

MAO monoamine oxidase

MC mast cells

MCP monocyte chemoattractant protein

MHC major histocompatibility complex

MIP macrophage inflammatory protein

MRI magnetic resonance imaging

NADPH nicotinamide adenine dinucleotide phosphate

NANC nonadrenergic noncholinergic

NGF nerve growth factor

NK natural Killer

NKX homeodomain-containing transcription factor

NLR neutrophil to lymphocyte ratio

NLR NOD-like receptors

NO nitric oxide

NOD nucleotide-biding domain

NSE neuron specific enolase

PAMP pathogen-associated molecular pattern

PAR protease-activated receptors

PCR polymerase chain reaction

PFA platelet activating factor

PGP protein gene product

PLR pattern recognition receptor

PRR pattern recognition receptors

RANTES (protein) regulated on activation, normal T cell expressed and secreted

RIF right iliac fossa

SD standard deviation

SERT serotonin transporter

SNP single nucleotide polymorphism

SP substance P

TCR T cell receptor

TF tissue factor

TGF transforming growth factor

Th T-helper

TLR toll-like receptors

TNBS trinitrobenzenesulfonic

TNF tumour necrosis factor

TRPV transient receptor potential ion channels of the vallinoid

UC ulcerative colitis

VIP vasoactive intestinal peptide

WCC white cell count

CHAPTER 1: Introduction

1.1 The human immune system

The human immune system comprises humoral and cellular components, which play a vital role not only in the protection of the host from invading pathogens but also in the recognition and elimination of damaged or malignant-transformed cells. Antibodies, cytokines and the complement system constitute the main elements of the humoral component. The different leukocyte subsets constitute the cellular component.

The leukocytes, erythrocytes and thrombocytes are derived from the same progenitor cells (haematopoietic stem cells) in the bone marrow. Two common specific progenitors, which are the common myeloid and the common lymphoid progenitor cells, are derived from pluripotent stem cells. The myeloid lineage gives rise to erythrocytes, thrombocytes, mast cells, granulocytes (neutrophils, eosinophils and basophils), monocytes, macrophages and dendritic cells following development and differentiation in the bone marrow. The lymphoid lineage is developed into lymphocytes, which are distinguished by their site of maturation and subsequent differentiation. B lymphocytes develop and mature in the bone marrow whereas T lymphocytes and natural killer (NK) cells develop and mature in thymus.

The immune system comprises two distinct components, the innate and adaptive immunity. The innate immune component includes cellular-epithelial barriers (skin, mucosa), soluble components (complement system proteins, cytokines, chemokines, acute-phase proteins) and specific innate cell subpopulations (monocytes,

macrophages, neutrophils, dendritic cells, NK cells, platelets). The surface of the innate immune cell subsets possesses germ line-encoded receptors called pattern recognition receptors (PRRs). Some examples of PRRs are the toll-like receptors (TLRs), nucleotide-biding domain (NOD)-like receptors (NLRs) and retinoic-acid-inducible gene 1 receptors (PLRs). PRRs have the ability to recognise a variety of pathogen structures [pathogen-associated molecular patterns (PAMPs)] and trigger an immediate response. These receptors can also recognise products of damaged, injured or stressed host cells [danger (or damage)-associated molecular patterns (DAMPs)] such as heat shock protein (HSPs), S100 proteins, DNA, human group box protein 1 (HMGB1). Unlike the innate immune system, the adaptive immune system utilises a large selection of receptors encoded by rearranging genes to recognise a range of specific pathogen molecular structures. The adaptive immune system also displays immunological memory for previously encountered pathogens which confers a long-lasting immunity. Nevertheless, there is a delay in the initiation of an adaptive immune response due to the period of time that is required for the small number of specific B and T lymphocytes to undergo clonal expansion and become effector cells [B lymphocytes (plasma cells) producing antibodies, cytotoxic T lymphocytes (CTLs, T helper (Th) cells] to specifically remove the targeted antigen. Therefore, the innate immunity mediates the immediate and early immune responses, while the adaptive immunity mediates late and specific immune responses. However, the removal of an antigen is the collaborative result of the interaction between the innate and adaptive immunity. Cells and cytokines of the activated innate component of the immune system make a significant contribution to optimal activation of the adaptive component of the immune system.

Macrophages act as antigen presenting cells (APCs) and have a vital role in facilitating the immune responses to antigens. A summary of the main functions of the cellular and soluble components of the innate and adaptive immune systems can be found in Tables 1.1 and 1.2 respectively.

The lymphatic organs are an integral part of the immune system. They are composed of lymphoid tissue organised into anatomical and functional compartments containing lymphocytes as well as other immune cells. The primary or central lymphoid organs, which are the bone marrow and the thymus, generate lymphocytes from common lymphoid progenitor cells. The secondary or peripheral lymphoid organs which include the lymph nodes and the spleen, maintain mature naïve B and T lymphocytes and initiate adaptive immune responses. A series of different compartments can be identified within the secondary or peripheral lymphoid organs. The lymph nodes and the spleen respond to antigens that have entered the blood stream or the tissues and travelled via the lymphatics. The mucosal immune system [mucosal-associated lymphoid tissue (MALT)], part of which is the gut-associated lymphoid tissue (GALT), responds to antigens that attempt to breach the mucosa barriers [1-4].

Table 1.1 Innate immune system: Components and functions

Innate immune system	
Cellular components	**Principal functions**
Epithelial cells	Physical barrier
Neutrophils	Phagocytosis, killing of bacteria
Basophils	Killing of parasites, release of histamine
Eosinophils	Killing of antibody-coated parasites
Mast cells	Release histamine
Natural killer (NK) cells	Killing of virus-infected/malignant-transformed/damaged cells
Macrophages	Phagocytosis, killing of bacteria, antigen presentation
Dendritic cells	Phagocytosis, killing of bacteria, antigen presentation
Humoral components	**Principal functions**
Complement system	Opsonisation, killing of antibody-coated pathogens (classical pathway), chemotaxis
Cytokines	Induce, enhance or inhibit cellular responses
Chemokines	Act as a chemoattractant to direct the migration of cells, recruit monocytes, neutrophils and other immune cells from blood to affected tissue
Acute-phase proteins	Destroy or inhibit growth of microbes, affect coagulation, recruit immune cells to inflammatory sites, downregulate inflammation

Table 1.2 Adaptive immune system: Components and functions

Adaptive immune system	
Cellular components	**Principal functions**
Dendritic cells	Antigen presenting cells (APCs)
T helper (Th) cells	Modify immune responses: Th1 cells activate macrophages, CTLs and NK cells Th2 cells activate B cells, provide immunological memory Th17 cells mediate inflammation and autoimmunity through the CTL response in cancer and autoimmune diseases
Cytotoxic T lymphocytes (CTL)	Induce apoptosis, provide immunological memory
T regulatory cells (Tregs)	Inhibit generation of CTL responses, downgrade activated T lymphocytes, prevent autoimmunity by inhibiting self-reactive CTLs
B lymphocyte/plasma cells	Production and release of antibodies, induction of immunological memory
Humoral components	**Principal functions**
Antibodies	Opsonisation, neutralisation and complement activation
Cytokines	Induce, enhance or inhibit cellular responses

1.2 Gastrointestinal environment and inflammation

1.2.1 Overview

The gastrointestinal tract (GI) has the largest surface area between the body and the outside environment [5]. It is widely accepted that it does not only play a vital role in the digestion and absorption of food, but it also contributes to homeostasis [6, 7]. It is believed that it accommodates 1000 different types of bacteria with a total number of approximately 10^{14}. This number is equal to ten times the total number of the cells of the human body and can weigh up to 2 kg, making the human GI tract one of the most immensely populated microbial environments on the planet [7-10]. Therefore, the mucosa of the GI tract has to be able to not only tolerate a vast number of potentially harmful bacteria and dietary antigens, but also to recognise and even attack small numbers of infrequently intruding pathogens [5, 11]. On the other hand, GI microbes, whose collective number of genes is 100-fold greater than the total human genome [6, 8, 9], play a pivotal role in nutrition, metabolism, host defence and immune system development [6, 12]. However, the GI tract, in order to harness the benefits and also overcome the challenges of possessing such a large population of commensal bacteria, has evolved into the largest and most complex immune organ in the entire human body. It also possesses a widespread system of secondary lymphoid organs and a mammoth number of lymphocytes [5, 11, 13]. Thus, the GI immune system is crucial in regulating the dynamic interaction between host immunity and microbes with the aim to maintain homeostasis [5]. This fine balance is mediated by several cell populations, with some of them being unique to the GI tract, that following activation can initiate a series of immune functions. Failure to

preserve immune homeostasis will result in either acute or chronic GI inflammation [11, 14].

1.2.2 Anatomy of the GI mucosa

The GI tract functions as a dynamic barrier that has the ability to govern the movement of several substances between the lumen and the body. It allows the passage of water, electrolytes, and dietary substances but resists a potentially harmful invasion of commensal flora and their toxins [15, 16]. The main component of this barrier is characterised by the epithelial surface which comprises different populations of cells that are called intestinal epithelial cells (IEC) and include absorbent enterocytes, goblet cells, Paneth cells, neuroendocrine cells, dendritic cells and intraepithelial lymphocytes [6, 17]. The IECs form a single, polarised columnar layer that separates the intestinal flora from the underlying *lamina propria* [11, 18]. The mechanical cohesion between these cells and the control of paracellular permeability are safeguarded by different types of protein complexes forming tight junctions, adherence junctions and desmosomes [6, 15, 16]. Moreover, the IECs can also function as signal transducers due to their ability to sense luminal contents and release regulatory mediators that can initiate an appropriate response in the *lamina propria* [19]. The epithelial barrier is supported by a protective layer of mucin that prevents intestinal flora from invading the mucosa. This mucin is secreted by goblet cells and is primarily formed by glycoproteins. The colon has a dual layer of mucin, with the inner being much denser compared with the outer layer, preventing microbial motility and adhesion to the epithelium [11, 20, 21]. The dynamic intestinal mucosa is further supported by antigen-presenting cells (APCs) such as macrophages

and dendritic cells. Intestinal macrophages, characterised by the lack of CD14 expression, have been shown to exhibit phagocytic and bactericidal function [22]. Moreover, they can limit pro-inflammatory responses by suppressing Th1 and Th-17 immune reactions as well as produce anti-inflammatory cytokines e.g. IL-10 [23]. However, it has been shown that in patients with Crohn's disease macrophages can express pro-inflammatory cytokines such as IL-23, TNF-α and IL-6 [24]. Dendritic cells apart from being antigen-presenting cells that can stimulate T cells and promote their differentiation into inflammatory phenotypes can also prevent T cell mediated colitis as well as promote intestinal homeostasis [25-28].

1.2.3 Gut-associated lymphoid tissue

The GI tract is supported by different types of lymphoid organs that are jointly called gut-associated lymphoid tissue (GALT) [5]. It has been estimated that the intestinal lymphoid tissue constitutes almost one-quarter of the intestinal mucosa which means that approximately 70% of the entire population of immunocytes in the human body is found in the GI tract [29]. Peyer's patches and the isolated lymphoid follicles (ILFs) are closely associated with the mucosa, whereas mesenteric lymph nodes (MLN) are found in the mesentery [5]. Peyer's patches are well defined secondary lymphoid organs that are found in the small bowel and comprise 200-400 lymphoid follicles with each one consisting of a germinal centre surrounded by B and T lymphocytes and APCs which are further surrounded by follicle associated epithelium (FAE) containing M cells [29]. On the other hand, ILFs can be found along the entire intestinal tract [30, 31]. ILFs have similar anatomy to the lymphoid follicles of the Peyer's patches but they are highly dynamic as their number increases during

inflammatory responses and stimulate synthesis of IgA according to changes in the intestinal flora [5, 31-34]. Harmful bacteria or disruption of the epithelial barrier can activate dendritic cells, promoting their transport to MLN where they trigger differentiation of naïve T lymphocytes into effector and regulatory T lymphocytes. Therefore, the cytokines expressed by dendritic cells are able to drive the differentiation of naïve CD4+ T lymphocytes towards T helper (Th) 1, Th2, Th17 or regulatory T lymphocyte subsets [35]. Hence, GALT has evolved to such a degree that it is not only able to rapidly defend against invading bacteria but also to limit the inflammatory response to the gut environment while it allows the mucosa to efficiently absorb nutrients [31, 33].

1.2.4 Causes of GI inflammation

1.2.4.1 Overview

Several causative factors have been identified to be able to trigger an inflammatory response in the gastrointestinal track. Although the different factors could be broadly categorised in five broad categories as discussed below (disruption of the barrier, defective immunoregulatory mechanisms, intestinal flora, overexpression of proinflammatory factors and inappropriate host immune response), they frequently co-exist or more than one is required for the inflammatory response to result in clinical manifestations.

1.2.4.2 Disruption of the barrier

Disruption of the epithelial barrier in experimental models of intestinal inflammation has been demonstrated to be an important causative factor [36-38]. Panwala *et al.*

[37], using mice deficient for the Multiple Drug Resistance Gene 1a (mdr1a), showed that a defective non-immune component of the mucosal barrier can lead to inflammation of the colon. It is known that mdr1a is expressed by the colonic epithelial cells where it pumps toxins administered orally actively into the intestinal lumen [39]. Irradiated mdr1a deficient mice reconstituted with wild-type bone marrow spontaneously developed colitis, whereas, irradiated wild-type mice reconstituted with mdra1a-deficient bone marrow cells did not develop colitis. This study demonstrated that defective epithelial cells were responsible for the susceptibility to colitis in mdr1a deficient mice and not malfunctioning immune cells. The authors also demonstrated that the inflammatory response had features of ulcerative colitis and was characterised by dysregulated epithelial cell growth, ulcerations, crypt abscesses and lymphocytic infiltration of the mucosa. Interestingly, it was shown that oral administration of broad spectrum antibiotics could prevent colitis in mdr1a deficient mice which means that intestinal flora plays a vital role not only in the initiation but also in the perpetuation of colitis [37].

1.2.4.3 Defective immunoregulatory mechanisms

Defective immunoregulatory mechanisms have been implicated in the pathogenesis of intestinal inflammation. Chronic enterocolitis was identified in IL-10 deficient mice at the age of 4-8 weeks and was lethal for most animals. The presence of inflammatory infiltrates in the intestinal mucosa, fibrinoid and IgA deposits as well as abnormal expression of MCH II suggested a significant role of the immune system in the pathogenesis of the enterocolitis. IL-10 is known to be able to suppress cytokine synthesis in macrophages, natural killer (NK) cells and T lymphocytes. Therefore,

since the intestinal lumen is full of foreign antigens originating from food and bacteria that keep the intestinal immune system in a constantly active status, intestinal inflammation in IL-10 deficient mice could indicate a loss of control of the typical intestinal immune responses against intestinal pathogens leading to chronic inflammation due to overproduction of TNF-α, IL-1 and IFN-γ [40]. It was also demonstrated that the intestinal inflammation also perpetuated by the chronic overexpression of cytokines as administration of IL-10 temporarily cured the disease. Moreover, it was noted that the IL-10 deficient mice developed a less severe disease when kept in an environment with a defined microbial population. This finding suggested that the immune response in IL-10 deficient mice was prompted by microbial antigens, the composition of which determined the severity of the inflammation [40].

Moreover, deficient production of TGF-β has been shown to lead to colonic inflammation. TGF-β is a pleiotropic cytokine that can exhibit both anti-inflammatory and proinflammatory functions [41, 42]. Mice with TGF-β deficiency exhibited widespread inflammation in multiple organs [43, 44]. It was noted that this inflammation was not more prominent in mucosa than other tissues. Moreover, disruption of TGF-β signalling has been implicated in the development of inflammation in the colon amongst other organs [45, 46]. The development of colitis suggests that TGF-β has also been implicated in the regulation of epithelial cell function and in its absence the colonic mucosa is prone to stimulation by microbial antigens [46].

1.2.4.4 Intestinal flora

Several studies have implicated the mucosal microflora in the pathogenesis of GI inflammation not only in the initiation of the inflammation but also in the perpetuation of the inflammatory process. Sadlack *et al*. [47] demonstrated that IL-2 deficient mice developed aggressive ulcerative colitis-like colitis when kept under conventional conditions. However, when the mutant mice were bred in a germ-free (GF) environment they did not show any clinical or histological features of colitis [47]. However, when they were transferred to a facility with restricted and defined microbial environment (SPF) the mutant mice developed mild inflammatory bowel disease evident on histological and immunological analysis [47]. These results strongly suggested that colitis was a direct consequence of an abnormal response of the mucosa to the intestinal bacterial flora. Similar results were reported in a study conducted by Contractor *et al*. [48]. However, the authors attributed the mild inflammation and anaemia seen in the SPF IL-2 deficient mice to uncontrolled responses to endogenous and not to environmental antigens. On the other hand, Schultz *et al.* [49] showed that older IL-2 deficient mice suffered from of mild, focal and subclinical GI inflammation even when they were bred in GF facilities. Nonetheless, SPF mutant mice showed a much more severe clinical picture confirming the vital role of intraluminal bacteria in the pathogenesis of GI inflammation [49].

In addition to the aforementioned studies, further evidence that implicated the bacterial flora in the GI inflammation originated from studies demonstrating that T lymphocytes in the mouse *lamina propria* did not react against their own intestinal

flora but they did react to the microflora of other mice even from the same strain [50, 51]. This tolerance towards self-antigens was both local and systemic and it was disrupted by a model of experimental colitis [trinitrobenzenesulfonic acid (TNBS)] where mice lost their non-responsiveness to their own flora but they regained it when the colitis was resolved [51]. This evidence suggested that colitis was at least partially caused by the foreign antigens of intestinal flora, either due to cross reactivity to TNBS or loss of tolerance to intestinal antigens with the onset of colitis. The loss of tolerance to self-microbial antigens was also evident in human inflammatory bowel disease (IBD), suggesting that the human disease can also be attributed to the loss of tolerance to self-flora [50-52].

1.2.4.5 Overexpression of proinflammatory factors

Another important factor that it has been shown to cause GI inflammation is the overexpression of proinflammatory cytokines such as TNF-α [53]. Kontoyiannis *et al.* [53] using a TNF DARE mouse model that was characterised by disrupted regulation of the TNF-α mRNA processing which resulted in overproduction of TNF-α, demonstrated that these mice developed a notable mucosal inflammation in the terminal ileum and proximal colon. The colitis resembled the features of human Crohn's disease as there was transmural involvement and the infiltrative lesions contained rudimental granulomata resembling non-caseating granulomas with multinucleated giant cells. It was also shown that the colitis was dependent on the presence of T lymphocytes as well as constituents in the bacterial flora that could stimulate the production of TNF-α. Nevertheless, TNF DARE mice that were also IL-12 p40 deficient did not develop colitis which implied that microflora antigens did not

directly induce TNF-α production in the absence of IL-12. IL-12 production is upregulated in TNF ΔARE mice as TNF-α overexpression leads to the development of a positive feedback loop between IL-12 and TNF-α [53].

Several studies also demonstrated that IL-23 could promote chronic intestinal inflammation by inducing a wide range of inflammatory responses. It has been shown that T lymphocyte-mediated colitis was associated with overexpression of proinflammatory cytokines such as TNF-α, IFN-γ, IL-6 and IL-17A whose expression was significantly downregulated in the absence of IL-23 [54-56]. Analysis of mucosal CD4+ T lymphocyte responses in both studies published by Hue *et al.* [54] and Kullberg *et al.* [55] has shown that IL-23 dependent T lymphocyte-mediated colitis was associated with strong Th1 responses with a minor IL-17-expressing cells being present. Nevertheless, in the presence of anti-IL-23p19 antibodies the colitis that was induced by adoptive transfer of a bacteria reactive CD4+ Th17 cell line, was ameliorated. This finding was associated with increased apoptosis of the Th17 cells [56]. Interestingly, with complete absence of T lymphocytes, the innate immune typhlocolitis triggered by *H. hepaticus* infection of 129RAG-/- mice was characterised by very similar expression of pro-inflammatory cytokines as found in the T lymphocyte mediated colitis, which was also attenuated by administration of anti-IL-23p19 antibodies [54]. This type of innate immune response was characterised by the presence of granulocytes and monocytes that were expressing IL-17A [54]. Therefore, there is evidence to suggest that IL-23 can induce the expression of proinflammatory mediators in both acute innate and pathogenic adaptive responses, which could lead to the development of chronic intestinal inflammation.

1.2.4.6 Inappropriate host immune response

Finally, inappropriate immune response of the host to an external factor has been shown that could result in GI inflammation. Among the common causes of non-infectious GI inflammation are the celiac disease and IBD that includes ulcerative colitis and Crohn's disease. Coeliac disease is characterised by the presence of small bowel mucosal injury and nutrient malabsorption. It is found in genetically susceptible individuals following dietary ingestion of proline and glutamine rich protein that are widely called gluten. The symptoms often settle with adoption of gluten free diet [57]. Acquired T lymphocyte-mediated immune mechanisms and innate immune mechanisms play a significant role in the pathogenesis of celiac disease [58]. The main genetic risk factor is a susceptible HLA haplotype, while numerous other risk-associated loci map to genes involved in immune function such as chemokines and cytokines have been implicated in the pathogenesis of celiac disease [59].

The pathogenesis of IBD has also been shown to have a significant genetic component and is believed to be associated with a dysregulated immune response to intestinal flora. Polymorphisms in genes of the innate immune system such as NOD2 (which encodes nucleotide-binding oligomerisation domain 2), ATG16L1 (which encodes autophagy related 16-like protein 1) and IRGAM (which encodes immunity-related GTPase family M) are specific to patients with Crohn's disease but have not been described in ulcerative colitis patients. On the other hand, several gene alterations in the IL-23 pathway, such as IL-23 receptor, IL-12B (which encodes the p40 subunit of IL-12 and IL-23) and STAT3 (which encodes the signal transducer and activator of

transcription 3) have been described in both ulcerative colitis and Crohn's disease. Moreover, the homeodomain-containing transcription factor NKX2-3 has also been implicated in the pathogenesis of both ulcerative colitis and Crohn's disease as studies in NKX2-3-deficient mice have demonstrated its role in the correct localisation of lymphocytes in the spleen for both B lymphocyte maturation and T lymphocyte immune responses [25, 35, 60-63].

Therefore, it is apparent that in the complexity of the intestinal environment, gastrointestinal inflammation can often be multifactorial as several causative factors need to coexist for the inflammatory response to have a harmful effect to the host and consequently to become clinically apparent. Additionally, the enteric nervous system and the neuroimmune interactions also play a crucial role in the regulation of immune responses within the gastrointestinal inflammation.

1.3 Enteric nervous system and neuroimmune interactions

1.3.1 Overview

There is increasing evidence on the involvement of the enteric nervous system (ENS) in the enteric immunology and regulation of the inflammatory process [64-66]. Almost any inflammatory process in the GI tract is associated with abdominal pain as products of the immune system sensitise and stimulate peripheral sensory neurons following tissue damage [67, 68]. It has been reported that 50%-70% of patients with IBD present with abdominal pain [69, 70]. Moreover, patients with IBS can present with a variety of functional GI disorders but mainly with abdominal pain and altered bowel habits [67, 71].

1.3.2 Innervation of the GI tract

The wall of the GI tract contains approximately 10^8 neurons which are divided into subpopulations of sensory, interneurons and motor neurons. They are interconnected with synapses into integrated circuits forming the myenteric and the submucosal plexuses [72-74]. The submucosal plexus mainly supplies the mucosa and controls secretion, absorption and blood flow whereas, the myenteric plexus principally controls motility [75, 76]. The connection to the CNS is provided by extrinsic primary afferent nerve fibres of the GI tract which receive splanchnic and vagal innervation along with nerve fibres from the pelvic nerves [77]. Although the splanchnic afferents constitute the main nociceptive pathway, vagal as well as pelvic nerves have been associated with the preservation of homeostasis and nociception of the gut.

The vagal afferents primarily control reflexes such as gastric emptying, pancreatic secretion and the perception of bloating and nausea. Three types of vagal fibres have been identified: mechanical, tension sensitive and chemo-sensitive afferents [78-82]. The pelvic afferent fibres respond to colonic distension and hence they transmit information on physiological sensation such as urgency and desire to defecate [83]. Animal studies have implicated the pelvic afferents in acute and inflammatory pain as bilateral pelvic nerve transection eliminated pain associated behaviour [77, 83] and administration of chemical irritant to colonic tissue caused sensitisation of the pelvic afferents [84]. The splanchnic afferents act as functional complement to the vagal and pelvic fibres and they are considered to be the main nociceptive pathway as they conduct different modalities of mechanosensory information [83, 85]. The vast majority of the splanchnic afferents are found in the serosa and mesenteric membranes in close association with mesenteric blood vessels. It is believed that the mesenteric and submucosa extrinsic fibres are able to respond to distortion of the intestine due to contraction or distension at levels that could cause pain [83, 86, 87]. However, the relevance of the extrinsic afferent nerve fibres to pain is due to their stimulation by neuropeptides [64].

Although, the large intestine is extensively innervated, a small number of extrinsic afferents provides the link of with the central nervous system (CNS) for perception. Moreover, the extrinsic colonic afferents supply other organs by organising into complex plexuses distributed throughout the abdominal cavity. Therefore, signals from multiple abdominal organs, and not only from the colon, congregate to a comparatively large number of spinal cord segments [88]. Within the spinal column

somatic input also joins the visceral signals and together travel to CNS. This equivocal signal transduction from the gut to the brain could explain the poor topological relation of the experienced pain in many intra-abdominal conditions which is often described as "non-specific" by clinicians.

1.3.3 Neuroimmune interaction in the GI tract

The ENS closely cooperates with the enteric immune system in order to establish a first line defence against noxious agents. Several studies have shown that different cell types are in close histoanatomic proximity with enteric neurons, vagal nerve fibres and spinal sensory nerves in order to be able to regulate the immune response in different ways [89-91].

Enterochromaffin cells are neuroendocrine cells that are strategically positioned between the epithelial cells of the GI mucosa where they can sense the intraluminal milieu. They are equipped with secretory granules that contain a wide range of peptides, such as serotonin, cholecystokinin and secretin that can influence peripheral nerve fibres upon their release [92]. Several studies have implicated enterochromaffin cell hyperplasia in the pathogenesis of IBS and IBD [65, 93, 94] as well as in Th2 inflammatory response [95].

Mast cells are also an integral part of the innate immune system and play a crucial role in neuroimmune interactions [64, 96]. They have the ability to act as sensory cells activated by different effector cells and release a wide range of active mediators such as histamine, serine proteases, proteoglycans as well as prostaglandins, leukotrienes

and cytokines (TNF-α, IL-6) [64, 96]. The release of nerve growth factor (NGF) by mast cells is stimulated by IL-1β and TNF-α and it can not only excite the afferent neurons but also increase neuropeptide expression from the spinal cord sensory terminal and is likely to contribute to centrally mediated hyperalgesia [64, 97-99]. Moreover, NGF increases neuropeptide expression at peripheral nociceptors terminals and proteolytic products of NGF in inflamed tissues can indirectly modulate their excitation [97, 100]. Therefore, mast cells can control sensory terminals by modifying their sensitivity and amplitude via the release of NGF. As a result, the degree of mast cells activation has been directly associated with hyperalgesia and pathological pain status in inflammatory conditions [97].

Another population of cells that contributes to the neuroimmune interactions is the peripheral glial cells. They are able to control enteric neurotransmission, inflammation and intestinal barrier function as they contain neurotransmitter precursors such as γ-aminobutyric acid (GABA) and nitric oxide; express purine receptors and produce cytokines (IL-1, IL-6, TNF-α), NGF and neuropeptides (NKA and Substance P) following their activation [101]. With regards to neurotransmission, it has been shown that GABA transporter GAT2 is mainly expressed by enteric glial cells which suggests that glial cells are implicated in the detoxification of GABA via rapid GABA-removal from the extracellular space [102]. Moreover, partial ablation of enteric glial cells in an animal study showed decreased expression of neuronal VIP and NO, further supporting the role of the glial cells in peptidergic and nitrergic neurotransmission [103].

The enteric glial cells also play a pivotal role in both neuroimmune interactions and the function of intestinal barrier. They have been shown to promote intestinal seal by releasing soluble factors that interact with the epithelial cells. Ablation of the enteric glial cells caused severe colonic inflammation due to breakdown of the epithelial barrier. On the other hand, it has also been described that glial cells can inhibit the proliferation of the intestinal epithelial, suggesting their role in preventing disorganisation and hyperproliferation during an inflammatory process [104-107]. The seeming paradox between epithelium conserving and anti-proliferation effects could be explained by the observation that glial cells were able to change the arrangement of the epithelial cells which flattened and spread out in order to enhance epithelial sealing [104, 105, 108] as the intestinal epithelial barrier is a vital part of the innate immune system [101, 109, 110]. Moreover, enteric glial cells play an important role in attracting inflammatory cells as it has been reported that lymphocytic infiltrates in the ENS of patients with Crohn's disease are associated with the expression of major histocompatibility complex (MHC) class II on glial cells [111-113]. Moreover, co-culture of enteric glial cells, stimulated by TNF-α and interferon (IFN) γ in the presence of an antigen and antigen-specific T lymphocytes initiated a substantial increase in T lymphocyte proliferation [101]. Thus, it is apparent that enteric glial cells are a fundamental part of the enteric immune system as well as the ENS and play a pivotal role in GI inflammation.

In conclusion, the ENS in conjunction with several cell populations is in a position regulate immune responses. Release of neuropeptides such as Substance P, CGRP and VIP can initiate neurogenic inflammation [114]. Release of Substance P

stimulates mast cell degradation which causes the release of different inflammatory mediators such as cytokines, proteinases, nitric oxide, prostaglandins, leukotrienes and thromboxanes [114]. These substances subsequently activate other inflammatory cells such as neutrophils, lymphocytes and macrophages whose mediators in turn engage in further neuroimmune interactions stimulating the ENS even further and the final result is an amplified the inflammatory response [115].

CHAPTER 2: Immunological characterisation of inflammatory, neuroimmune and histologically "normal" appendicitis

2.1 Overview

Acute appendicitis is the most common surgical condition that requires an urgent abdominal operation. The number of hospital admissions in England has been reported to exceed 40000 per year and is more common in males with a male to female ratio of 1.4:1 [116]. The lifetime risk is 8.6% in males and 6.7% in females in the USA [116]. The diagnosis of appendicitis is primarily clinical as it is based on the history and physical examination. Although, it is fairly easy to diagnose cases with classical presentation, this is not always the case and, therefore, the diagnosis can be very challenging. Moreover, it is widely accepted that delay in treatment carries significant risk and increases post-operative complications [117]. This has led clinicians to perform early appendicectomy in patients with suspected appendicitis; an approach that has resulted in a reduction of the morbidity. The consequence of this practice is that a significant number of appendices are reported as normal following conventional histopathological examination [118, 119]. Before the laparoscopic era, the rate of histologically "normal" appendices was reported to range from 15% to 30% [120, 121]. The introduction of diagnostic laparoscopy in the early 1990s has reduced the rate of unnecessary appendicectomy [122, 123]. However, even using laparoscopy as a tool to further investigate right iliac fossa pain the dilemma of removing a macroscopically normal appendix still remains. As there are studies either supporting [124, 125] or opposing [126] the removal of a normal looking appendix, the decision rests with the surgeon’s discretion. Despite the frequency of occurrence, the pathogenesis of acute appendicitis remains poorly

understood. The fact that the clinical presentation is conflicting with the absence of definite histological findings combined with the clinical observation that appendicectomy relieves right iliac fossa pain in most patients suggests that an unknown pathology may exist [127, 128]. Several studies have investigated the discrepancy between the clinical and histological diagnosis and have shown that not only the inflammatory process can vary considerably but have also highlighted different immunological and cellular markers that may be specific for the type and pathogenesis of appendicitis (inflammatory/neurogenic) [129-135]. More recently, it has been suggested that uncomplicated acute appendicitis may be managed successfully with antibiotics rather than surgery [136]. However, the long-term consequences of this approach have not been elucidated and it is not known whether patients treated with antibiotics may have chronic right iliac fossa pain as a consequence of neuroimmune remodelling.

2.2 Pathogenesis of appendicitis

Acute appendicitis was first described as a clinical entity by Reginald Fitz in 1886 who attributed its cause to indigestion, jumping, lifting and falling. Nowadays, obstruction of the appendiceal lumen is thought to be the cause for the development of acute appendicitis and subsequently appendiceal gangrene and finally perforation [137]. Overall, obstruction of the lumen can be due to a faecolith, fibrotic stricture, foreign body or indigested food. While, over-growth of the lymphatic tissue is a cause of acute appendicitis in the young, obstruction of the appendiceal orifice by a caecal carcinoma can lead to acute appendicitis in the elderly. Once the luminal obstruction is established, the intraluminal pressure increases due to accumulation of secreted mucus. As a consequence, the lymphatic drainage is obstructed leading to oedema and mucosal ulceration. At this stage of the inflammatory process, complete resolution may be possible either spontaneously or due to treatment with antibiotics However, in the case the condition progresses, bacterial proliferation continues, and the tension rises. The venous drainage is obstructed first followed by arterial supply. With the mucosa being ischaemic bacterial invasion takes place resulting in acute inflammation. Further progression of the acute inflammatory process in combination with compromised arterial supply leads to gangrene and perforation with subsequent bacterial contamination of the peritoneal cavity. However, in case the greater omentum and the small bowl become adherent to the appendix the acute inflammation can be walled off and the result is a phlegmonous mass which will eventually contain pus [137].

2.3 Literature review

2.3.1 Overview

Although the aforementioned mechanism is found in the vast majority of surgical textbooks, it cannot explain the symptoms of acute appendicitis in patients without features of acute inflammation on histological examination of their appendix. The cause of the right iliac fossa pain in these patients remains unknown and the clinical improvement after appendicectomy cannot be explained. In this context, several studies using laboratory-based techniques have tried to explain the role of inflammatory mediators and neuroproliferation in causing the clinical picture of acute appendicitis without the presence of acute inflammation on conventional microscopy [127, 129-135].

2.3.2 Neurogenic appendicopathy

The impression that the ENS could be responsible for symptoms mimicking acute appendicitis dates back to 1921, when Maresch [138] and Masson [139] independently described proliferation of the ENS giving rise to neuromatous lesions of the appendix without any features suggesting acute inflammation. Masson [139, 140] was the first to recognise the close spatial relationship between nerve fibres and endocrine cells in the appendiceal mucosa and suggested the term "*appendicite neurogene*" introducing the clinical entity of neurogenic appendicitis. However, this term was criticised by Knoflach [89] and therefore the term "neurogenic appendicopathy" was suggested by Chiari [90]. Feyrter suggested that abnormal communication between the "*adeno and neurointestinum*" can potentially cause

neurogenic appendicitis [91]. In a later study Feyrter also suggested that the pathophysiological mechanism responsible for irregular peristalsis, spasm and hyperemia of appendiceal blood vessels is a neurovascular crisis [141]. Aubock and Ratzenhofer [142] with the use of electron microscopy were able to demonstrate the presence of enterochromaffin cells in the *lamina propria* of the appendicular mucosa and their direct contact with normal or pathologically altered nerve fibres confirming Masson's early theory. In specimens with features of neurogenic appendicopathy the number of enterochromaffin cells was increased [142]. In 1980s, Hofler [143, 144] suggested the following classification based on nerve proliferation and axonal swelling in the mucosa as it was observed by Aubock and Ratzenhofer [142, 145] with the use of electron microscopy:

1. Mucosal neurogenic appendicopathy is the most frequent type with an incidence of approximately 10.4% of all excised appendices.
2. Central or axial neuromas occur following obliteration of the proximal appendix and result in a large central nerve bundle, surrounded by the muscularis mucosae. In both types the number of endocrine cells in the mucosa stroma is increased.
3. Neuromuscular proliferation is uncommon and is characterised by a striking proliferation of smooth muscle fibres and axons close to the mucosa or *muscularis propria* layers resulting in neurovascular tangles. Neuromuscular proliferation is not associated with inflammatory and endocrine cell proliferation.

The incidence of neurogenic appendicopathy seemed to vary significantly between studies depending on the study design. In an early study which was carried out by Doberauer [146] and examined patients older than 60 years of age, the reported incidence of neurogenic appendicopathy was 50%, whereas, Quell and Horvath [147] based their study on retrospective data and reported an incidence of 60%. Another study which was conducted on postmortem material and examined a series of 237 appendices for neurogenous hyperplasia using immunohistochemistry [148]. The specimens varied from appendices with intact lumens, featuring intramucosal neurogenous hyperplasia often with co-existent submucosal and muscular nerve growth, to obliterated samples whose axial portions were composed of nerve tangles and fibrous tissue. The neurogenic lesions in this study were found to be present in more than 80% of all the specimens [148]. The first prospective study which included 127 appendices confirmed neurogenic appendicopathy in 44.8% of the specimens without histological features of acute appendicitis whilst 14.5% of patients with acute appendicitis were also found to have features of neurogenic appendicopathy [149].

An additional study by Hofler *et al.* [150] extended previous knowledge demonstrating an increased number of nerve fibres in the mucosa of appendices of the mucosal type of neurogenic appendicopathy. The nerve fibres of the mucosal plexus and the innermost layers of the submucosa were also significantly enlarged as visualised by neuron specific enolase (NSE). A similar staining pattern was demonstrated by Substance P whereas nerves with VIP immunoreactivity were also numerous but to a lesser extent. It was suggested that Substance P could cause spastic contractions and abnormal peristalsis while VIP secretion could be implicated

in the mechanism of local hyperaemia. This proposition was in agreement with Feyrter's theory [91, 141] of a neurovascular crisis [150]. In support to this study, Naik [151] also reported mucosal and submucosal hyperplasia in 69.34% of the histologically "normal" appendices when stained with PTAH. In contrary to the findings of the previous studies [150, 151], Dhillon and Rode [152] reported that staining for NSE revealed more instances of nerve "hyperplasia" in the control group compared with appendices generating abdominal pain without active inflammation confuting the clinical entity of neurogenic appendicopathy based on quantitative nerve changes. However, the intensity of staining for serotonin in the subepithelial neuroendocrine cells was significantly reduced in the painful group suggesting discharge from serotonin stores. There were no differences between the two groups for VIP and Substance P staining. The authors hypothesised that continuous release of serotonin could lead to acute appendicitis [152]. However, both studies by Naik [151] and Dhillon and Rode [152] performed a subgroup analysis in patients with appendices without signs of acute inflammation.

Franke *et al.* [153] conducted a prospective multicentre study in an attempt to delineate preoperative clinical signs that could differentiate neurogenic appendicopathy from acute appendicitis. The authors also examined how reliable simple haematoxylin and eosin (H&E) staining was for the histological diagnosis of neurogenic appendicopathy. The study evaluated 282 appendices and neurogenic appendicopathy was diagnosed by H&E when a focal collection of pale spindle cells was found in the *lamina propria* or in an obliterated appendix. Immunohistochemistry with S-100 protein was performed to confirm diagnosis of

neurogenic appendicopathy. Clinical data was also collected and analysed. Regarding the histological diagnosis, 7 of the 19 patients with acute appendicitis were found to have features of mucosal neurogenic appendicopathy by both H&E and S-100 staining. H&E staining missed the diagnosis of neurogenic appendicopathy in only 7 of the 96 histologically " normal" appendices while they were detected by S-100. Therefore, the sensitivity of H&E in the histologically "normal" group was reported to be 84% with specificity 100%. With regards to clinical findings, evidence emerged that patients with neurogenic appendicopathy were more likely to have had similar previous episodes compared with acute appendicitis patients. There were no differences in the characteristics of the pain, nausea, micturition or gynaecological symptoms. Physical signs as well as laboratory tests such as leukocyte count to suggest acute inflammation were more frequent in the acute appendicitis group compared with the neurogenic appendicopathy group. Thus, this study provided evidence that neurogenic appendicopathy could be easily diagnosed by H&E staining but could not be differentiated from acute appendicitis based on the clinical picture [153].

However, a recent study by Grebeldinger *et al.* [154] not only confirmed the findings of Franke *et al.* [153] reinforcing the role of H&E staining in the diagnosis of neurogenic appendicopathy but also described clinical parameters that characterised neurogenic appendicopathy. This study included 209 individuals aged 4 to 19 with clinical diagnosis of acute appendicitis. The negative appendicectomy group consisted of two subgroups: with neurogenic appendicopathy and without neurogenic appendicopathy. The control group was formed by histologically proven

acute appendicitis specimens. A sample was diagnosed as neurogenic appendicopathy by H&E staining when a focal collection of pale spindle cells was found in the *lamina propria* mucosa or the lumen was obliterated. The diagnosis of neurogenic appendicopathy was confirmed with S-100 staining of the Schwann cells. The clinical examination findings were associated with information gathered by laboratory and ultrasonography tests. The follow-up of the patients lasted for three months after discharge. Thirty-eight % of the macroscopically normal appendices reported to have features of neurogenic appendicopathy while the highest incidence of neurogenic appendicopathy was at the age of 16 to 19 and most of the patients were female (n=14). Patients with neurogenic appendicopathy ware associated with the presence of previous episodes mimicking acute appendicitis and they were likely to have a characteristic antalgic position when in pain but unlikely to have guarding or a coated tongue with low or normal white cell count. Patients with neurogenic and non-neurogenic appendicopathy were less likely to have faecoliths compared with samples with evidence of acute inflammation. Intraabdominal abscesses were not detected in patients with neurogenic appendicopathy whereas free fluid in the right iliac fossa was detected in 36.8% of patients with neurogenic appendicopathy, 25.8% with non-neurogenic appendicopathy and 32.1% with acute appendicitis. Regarding the post-operative follow-up, the number of neurogenic appendicopathy patients with pain in the scar, persistent right iliac fossa pain or digestive disorders was smaller compared with the non-neurogenic appendicopathy or acute appendicitis groups. The reoperation rates were reported to be zero in the neurogenic appendicopathy group whereas 3.8% and 6.5% of patients with acute appendicitis and non-neurogenic appendicopathy groups respectively needed re-operation [154].

In a similar study, Sesia *et al.* [155] retrospectively examined 29 paediatric appendices that met the histopathological criteria of neurogenic appendicopathy. The intraoperative diagnosis for these 29 appendices was: absence of inflammation in 17.2%, acute inflammation in 58%, phlegmonous in 13.8% and perforated appendicitis in 10.4% of the specimens. The most common type of neurogenic appendicopathy was the submucosal as it was found in 52% of specimens, whereas the mucosal was found in 41% and the axial neuroma in 7% of the specimens.

The findings of the previously mentioned studies recognised neurogenic appendicopathy as a clinical and histopathological entity in patients who presented with signs and symptoms suggesting acute appendicitis and subsequently had "negative" appendicectomy. Some differences in the clinical picture of neurogenic appendicopathy and acute appendicitis were identified which suggested that the diagnosis of neurogenic appendicopathy should be considered in patients with right iliac fossa pain, especially in those with recurrent symptoms even if the appendix looks normal intraoperatively. However, these studies did not provide evidence on the status of the immune system of histologically "normal" appendices as well as on possible interactions between the ENS and the immune system.

2.3.3 Neuroimmune appendicitis

The term "neuroimmune appendicitis" was introduced by Di Sebastiano *et al.* [130] in 1999 implying that disturbed interactions between the ENS and immune cells were involved in the pathophysiology of acute right iliac fossa pain in the absence of acute inflammation of the appendix on conventional microscopy. The patients involved in

this study were recruited prospectively and allocated into three groups. The acute appendicitis group was formed by appendices with evidence of acute inflammation on conventional microscopy whereas appendices that did not have any of these signs formed the histologically "normal" group. The control group was formed by appendices obtained from multi-organ donors or patients who had their appendix removed as part of an elective abdominal surgery. With the use of immunohistochemistry, it was shown that in the control samples, plexuses of nerve fibres stained positively for Substance P extended from the submucosal layer to the *lamina propria* of the mucosal layer as well as beyond the outer zone of lymphoid follicles being in close contact with mononuclear immune cells. VIP staining showed a similar picture. In the acute appendicitis group, the staining for Substance P, VIP and GAP-43 was significantly reduced and proportional to the infiltration of immune cells. The histologically "normal" appendices showed significantly enlarged lymph follicles and well developed germinal centres with nerve fibres to be in contact with the marginal layer of the lymph follicles, but they were not detected in the centres. A significant increase of nerve fibres stained by Substance P and VIP was observed mainly in the basal part of the mucosal layer. Moreover, intrinsic and ganglionic cells in the myenteric and submucosa plexus showed strong staining for Substance P and also most of the nerve trunks and neurons showed immunoreactivity for GAP 43 and PGP 9.5. With the use of quantitative computerised image analysis of mucosal nerve-fibre density demonstrated that the area with Substance P immunostaining was proportionally greater in the histologically "normal" group compared with the acute appendicitis and control groups [130]. Interestingly, a significant number of patients in the group with histologically "normal" specimens, reported previous attacks of

pain whereas only one patient with histologically proven appendicitis had experienced right iliac fossa in the past. In the follow-up period, the vast majority of patients in the histologically "normal" group were pain free. The latter was in agreement with the findings of an earlier study according to which two thirds of patients with histologically "normal" appendix were pain free after a mean follow-up of 2.9 years [117].

The concept of neuroimmune appendicitis was further evaluated in a small retrospective study which involved 19 children allocated in three groups [134]. With the use of immunohistochemistry, Substance P was found to be moderately to strongly expressed in the epithelium, *lamina propria* and *muscularis* in more than 50% of the histologically "normal" samples. The expression of VIP demonstrated a similar pattern although it was almost absent in the epithelium. According to the authors the reported expression of neuropeptides in the histologically "normal" samples was analogous to that observed in specimens with acute inflammation. In the control group the expression of these neuropeptides was either negative or very weak. In contrary to Di Sebastiano *et al.* [130] the immunoreactivity for GAP-43 was very weak and non-specific. After a follow-up period of one year, the right iliac fossa pain was settled in all children with histologically "normal" appendices. In agreement with observations by other studies, Bouchard *et al.* [134] noted that some of these children experienced previous episodes of right iliac fossa pain.

The studies by Di Sebastiano *et al.* [130] and Bouchard *et al.* [134] clearly showed that neuroproliferation associated with increased expression of Substance P and VIP

neurotransmitters could cause neurogenic inflammation that gave rise to signs and symptoms of acute appendicitis without the presence of acute inflammation evident on conventional histological examination. Moreover, the increased expression of neuronal plasticity marker GAP-43 in the histologically "normal" group indicated that the ENS was grossly affected by the inflammatory process. Both studies also confirmed the presence of previous right iliac fossa pain episodes that settled with appendicectomy. However, several aspects on the nature of the neuroimmune interactions and they could regulate the inflammatory response remained to be explained.

In an attempt to further investigate the pathogenesis of appendicitis and more specifically the cause of symptoms in patients with histologically "normal" appendices, Xiong *et al.* [132] examined the possible association between the ENS and mast cells in patients with a clinical diagnosis of acute appendicitis. The study was retrospective in nature and included 40 paediatric appendices, 10 of which were reported to be histologically "normal". The expression of Schwann cell markers S-100 protein and 2’2’-cyclic nucleotide 3’ phosphodiesterase (CNPase) as well as neuronal markers synaptophysin and neuron-specific enolase (NSE) was assessed with immunohistochemistry. The number of mast cells was determined by counting the number of cells stained by mast cells tryptase. The acutely inflamed appendices demonstrated significantly increased number of nerve fibres and Schwann cells throughout the mucosa, submucosa, lamina propria and muscularis externa compared with the control group. On the other hand, 4 out of 10 histologically "normal" appendices demonstrated a very similar picture to the acutely inflamed

appendices with the rest 6 specimens being very similar to the control group. With regards to mast cells population, the acutely inflamed appendices showed a significantly increased number of mast cells in the *lamina propria* whereas the histologically "normal" appendices did not differ compared with the control group. However, in contrary to the results published by Xiong *et al.* [132], Naik *et al.* [156] also examined mast cell counts in inflamed and histologically "normal" appendices, and reported a significantly higher number of mast cells in the histologically "normal" samples.

Another study that attempted to investigate the alterations in the ENS and mast cells in patients with a clinical diagnosis of acute appendicitis but histologically "normal" appendices was conducted by Amber *et al.* [135]. The study included 50 specimens and immunohistochemistry with S-100 protein and NSE was employed to assess the status of ENS whereas mast cells were evaluated with toluidine blue staining. Conventional microscopy showed features of acute inflammation in 15 specimens whereas the rest 25 were reported as histologically "normal". Ten samples formed the control group. The authors reported that NSE stained fine nerve fibres that were located near the base and around the crypts of the mucosa. This finding was in contradiction with the findings of Xiong *et al.* who demonstrated fine nerve endings close to the epithelium and large nerve fibres near the bottom and between the crypts in 40% of histologically "normal" appendices. Neural hyperplasia limited to the mucosa only, as observed by Naik *et al.* [151] was found in only 4% of the histologically "normal" appendices. However, significant submucosal and myenteric neuronal hyperplasia in acutely inflamed and histologically "normal" appendices was

observed by Nemeth *et al.* [127], Xiong *et al.* [132] and Naik *et al.* [151]. In support of Di Sebastiano *et al.* [130] this study confirmed the presence of nerve fibres being in contact with the marginal zone of lymphoid follicles with the use of S-100 protein. Amber *et al.* [135] observed mast cells in all tissue layer with highest concentration in the submucosa of all groups contradiction. This finding was in contradiction to findings of previous studies according to which the highest concentration of mast cells was found in the mucosa [132, 156]. This discrepancy was attributed to the difficulty in the identification of mast cells in the mucosa due to enlarged lymphoid follicles [135]. However, the mean mast cell count was highest in the histologically "normal" appendices in all four layers compared with acutely inflamed appendices and control group and this was in agreement to the observation made by Naik *et al.* [156]. Xiong *et al.* [132] reported that the increased mast cell count observed in acutely inflamed appendices was associated with increased ganglia and Schwann cells whereas Amber *et al.* [135] reported that acute inflammation was associated with reduced mast cell count. This could be explained by a possible reaction of mast cells to luminal stimuli by elimination through the mucosa or degranulation and subsequent inability to detect them. Finally, in agreement with previous studies, 40% of the patients with histologically "normal" appendices reported to suffer from chronic abdominal pain [135].

The striking finding of neuronal hypertrophy in acutely inflamed appendices combined with a significantly increased number of mast cells could imply a close cooperation between the ENS and mast cells in the pathogenesis of acute appendicitis. However, the neuronal hypertrophy could be the consequence of a

chronic inflammatory process or repeated episodes of acute inflammation to stimuli. This was supported by the finding of increased nerve fibres and Schwann cells in histologically "normal" appendices from patients with signs and symptoms of acute appendicitis which might imply that these appendices represent a different degree of inflammatory response. The increased number of mast cells in both groups of acutely inflamed and histologically "normal" appendices could also support the idea that repeated episodes of inflammation could play a role to its pathogenesis. Moreover, the fact that a significant number of patients with histologically "normal" appendices reported previous episodes of right iliac fossa pain which eventually settled with appendicectomy, further strengthened the theory that neuronal hypertrophy could be the result of a chronic inflammatory process.

Nemeth *et al.* [127] confirmed the findings of previous studies which showed neuronal hypertrophy in histologically "normal" appendices by using whole-mount preparation using nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry and neuronal nitric oxide synthase (nNOS) immunohistochemistry. This technique gave the researchers the opportunity for an accurate 3D demonstration of the appendicular neuronal network, the relationship of branching nerve fibres to each other and neighbouring tissues as well as the changes in the neuronal plasticity. The study was conducted on paediatric specimens and retrospectively included 28 acutely inflamed appendices, 31 histologically "normal" appendices and 23 normal appendices which were removed during an elective abdominal surgery and formed the control group. Based on the density of the myenteric plexus two different subgroups were identified within the groups of

acutely inflamed and histologically "normal" appendices. In the acute appendicitis group 46.5% of the samples showed neuronal density similar to that of the control specimens whereas 58% of the histologically "normal" samples showed neuronal density similar to that of the control specimens. The rest of the samples were found to have thick nerve bundles and high-density neuronal network. The average nerve thickness of the histologically "normal" appendices was 101.1 µm whereas it was 96.7 µm and 58.4 µm in acutely inflamed and normal appendices respectively, clearly demonstrating significant changes in the innervation of histologically "normal" appendices obtained from patients with a clinical diagnosis of acute appendicitis. The authors agreed with the previously proposed theory and concluded that the observed neuronal hypertrophy is likely to have developed over a certain period of time due to repeated episodes of inflammation or chronic intraluminal obstruction [130, 135].

New insights into the pathogenesis of symptoms of appendicitis were brought by a study that examined the contents of neuroendocrine cells in histologically "normal" and acutely inflamed appendices [133]. The study involved 98 appendices from patients with a clinical diagnosis of acute appendicitis, 25 specimens had features of acute inflammation, 24 had evidence of follicular hyperplasia only and 26 specimens were reported to be histologically "normal" on conventional histopathological examination. The control group included 23 appendices resected from patients who had elective abdominal surgery. Enterochromaffin and subepithelial neuroendocrine cells were recognised with the use of immunohistochemistry for serotonin, chromogranin and synaptophysin. Immunoreactivity for serotonin and chromogranin was observed in the cytoplasm of enterochromaffin cells located at the base of the

crypts and in subepithelial neuroendocrine cells within the *lamina propria*, close to the base of the crypts. Synaptophysin was expressed by enterochromaffin and subepithelial neuroendocrine cells as well as by ganglion cells and the neural network within the *lamina propria* and between the muscle layers. Enterochromaffin cells were detected by serotonin and chromogranin staining in all the histologically "normal" and control appendices, 96% of the samples with follicular hyperplasia but in only 64% of the specimens with acute inflammation. The number of enterochromaffin cells per intact gland as well as the mean serotonin-containing cells per gland were significantly reduced in acutely inflamed specimens compared with the others. In a similar way, the number of chromogranin and synaptophysin immunoreactive cells were also significantly reduced in the acutely inflamed appendices compared with those of the other groups. Regarding the subepithelial neuroendocrine cells, they were stained by serotonin in 56% of acute appendicitis samples, 75% of follicular hyperplasia samples, 100% of histologically "normal" appendices and 91% of the control specimens. Immunostaining with synaptophysin and chromogranin did not demonstrate any significant difference between the four groups. Statistical analysis revealed that there was a strong correlation between the lack of neuroendocrine cells and acute inflammation.

The findings of this study were in agreement with several studies that observed a reduced number of enterochromaffin cells in clinical or experimental models of colonic inflammation [157-162]. However, the results regarding the subepithelial neuroendocrine cells were in contradiction with those reported by Dhillon and Rhode [152] who observed that the serotonin immunoreactivity was significantly reduced in

the subepithelial neuroendocrine cells of histologically "normal" but painful appendices compared with the control group. Nevertheless, the latter study did not investigate the serotonin content of inflamed appendices. Interestingly, in the group of follicular hyperplasia, Vasei *et al.* [133], observed a reduction in the number of enterochromaffin cells suggesting that this cell population is not only actively involved in the pathogenesis of acute right iliac fossa pain but it could also regulate the inflammatory process. Therefore, very early stages of inflammation like follicular hyperplasia could be transformed into acute inflammation due to sustained release of serotonin.

In conclusion, several studies demonstrated evidence that the ENS plays a crucial role in the pathogenesis of appendicitis as well as in the symptomatology of patients with histologically "normal" appendices. Strong experimental evidence suggested that neuronal hypertrophy is likely to develop over a certain period of time due to repeated episodes of inflammation or chronic intraluminal obstruction. This was supported by the fact that patients with histologically "normal" appendices reported several episodes of right iliac fossa pain which eventually settled with appendicectomy. Moreover, the ENS was shown to be involved in crucial neuroimmune interactions as Substance P and VIP were able to induce neurogenic inflammation explaining some of the signs and symptoms of appendicitis without evidence on an inflammatory process on conventional histology. The enteric nerve fibres were also shown to be in close proximity to mast as well as neuroendocrine cells which also played a significant role in the inflammatory process.

2.3.4 Immune system and histologically "normal" appendicitis

In 1996, Wang *et al.* [129] conducted the first study that demonstrated abnormal cytokine expression in histologically "normal" appendices obtained from patients with a clinical diagnosis of acute appendicitis. The study included 53 paediatric appendices, 31 of which were reported as histologically "normal" and employed in-situ hybridisation to assess the expression of TNF-α and IL-2 mRNA. The luminal obstruction as a cause of increased cytokine expression was excluded by selecting specimens without any evidence of faecolith in the appendiceal lumen. It was reported that seven of the 31 histologically "normal" specimens expressed TNF-α and IL-2 in germinal centres, submucosa and *lamina propria*. Although, the cytokine expression was not as strong as in the specimens with acute inflammation, it was very similar to those. The appendices of the control group showed almost complete absence of TNF-α and IL-2 mRNA expression.

In another study that tried to assess the inflammatory response in histologically "normal" appendices, Nemeth *et al.* [131] examined the expression of cyclooxygenase 1 and 2, prostaglandin E2, inducible nitric oxide synthase and major histocompatibility complex class II with immunohistochemistry using confocal laser microscopy. The study included 39 histologically "normal" appendices and 15 acutely inflamed appendices resected form children with a clinical diagnosis of acute appendicitis. Gangrenous and perforated appendices were excluded from the study. The study demonstrated increased expression of cyclooxygenase 2 (COX-2) in the epithelial cells of more than half of the histologically "normal" appendices. The level of COX-2 expression in these patients was the same as that of samples with acute

inflammation, whereas, it was undetectable in the control samples. Prostaglandin E2, iNOS and MHC Class II expression levels were also found to be strongly expressed in more than half of the histologically "normal" appendices.

The two studies provided evidence of an activated immune system at a stage where there were not any detectable inflammatory changes on conventional microscopy. The production of COX-2 is induced by cytokines such as IL-1 and TNF-α and it is responsible for the expression of prostaglandins, whereas, Prostaglandin E2 is considered as one of the most important proinflammatory prostanoid and a well-known mediator of hyperalgesia [131, 163, 164]. The study by Wang et al. [129] highlighted that the presence of localised abdominal pain in patients with suspected appendicitis could be attributed to an abnormal cytokine expression and not to an underlying luminal obstruction which has been considered as one of the principal causes of appendicitis. Even though an inflammatory process could be present at a molecular level without evident inflammatory infiltrates on routine H&E staining, the stage that the inflammatory process would reach and whether some other cytokines would be able to inhibit or amplify the inflammatory response remained unknown.

In a novel study, Murphy *et al.* [165] attempted to characterise the inflammatory gene expression in acute appendicitis. The study involved 18 paediatric appendices resected following a clinical diagnosis of acute appendicitis. The appendix specimens were divided into three groups based on the severity of inflammation as histologically "normal", mild and severe appendicitis. The study included only two samples that were histologically "normal". It was reported that the gene expression profiles of samples with severe and mild appendicitis was very similar. Although, the vast

majority of genes that was differentially expressed in mild appendicitis was also expressed in the severe appendicitis group, few genes that were differentially expressed in the mild group, were not differentiability expressed in severe appendicitis. Therefore, the study demonstrated that a core set of inflammatory genes were activated in all samples regardless of the degree of inflammation suggesting that there is a uniform inflammatory response underlying the pathological basis of appendicitis. The mild group could represent an earlier stage of inflammatory process or a less severe inflammatory response. However, none of the samples in the severe and mild appendicitis groups had faecoliths. On the other hand, the histologically "normal" appendices were found to have faecoliths. The gene expression in one of the two histologically "normal" samples was identical to that of the control samples and therefore the specimen was considered as a true normal appendix. Nevertheless, the gene expression in the second histologically "normal" appendix demonstrated both upregulation and downregulation of several gene clusters. As a result, the authors assumed that the inflammatory response in that sample could represent a very early stage most likely due to luminal obstruction. Additionally, independent analysis, demonstrated very low expression of TNF, IL-12, IFN-γ, IL-4, IL-2 and IL15 in acute appendicitis samples without significant difference compared to control samples. The authors concluded that there was no evidence of Th1 or Th2 inflammatory response. As the expression of IL-17 was also very low there was not any evidence to suggest a Th17 inflammatory response either. Despite the fact that both IL-1 and TNF are strongly induced by bacterial LPS, only IL-1β was induced with TNF expression being undetectable in the acutely inflamed samples. The researchers concluded that despite the impressive histological appearance of

acute appendicitis the underlying inflammatory response is very focused, suggesting the selective activation of mediators of the innate inflammatory response.

In conclusion, a small number of studies have provided evidence of upregulated expression of cytokines in patients who presented with right iliac fossa and their appendix was histologically "normal" on conventional microscopy. The abnormal expression of cytokines in this group of patients could indicate an activated immune system with an inflammatory response at very early stages with selective induction of specific inflammatory mediators. However, the inflammatory response could not be characterised as it was very focused and did not exhibit the features of any of the known inflammatory responses (Th1, Th2 or Th17). Therefore, more studies are needed to fully characterise the inflammatory profile of not only the histologically "normal" appendices but also that of the inflamed samples. Also, little is known about the immune response in complicated appendicitis as this group of patients is often excluded from the studies. Thus, the exploration of the potential differences or similarities in the immunopathogenesis of the complicated appendicitis compared with the uncomplicated would cover significant gaps in the existing knowledge.

Table 2.1 Consolidation of studies included in the literature review highlighting the investigated concept, study population, methodology and the studied markers.

Authors	Year	Concept investigated	Age group	Study population	Number of HNA samples	Method	Studied markers
Aubock and Ratzenhofer [142]	1982	Neurogenic appendicopathy	Adults & children	50	8	Electromicroscopy	ECC, nerve fibres
Olsen and Holck [148]	1987	Neurogenic appendicopathy	Adults & children	237	N/A	IHC	S-100
Wolf *et al.* [149]	1981	Neurogenic appendicopathy	Adults & children	127	4	H&E, clinical data	Clinical data
Hofler *et al.* [150]	1983	Neurogenic appendicopathy	Not stated	23	N/A	IHC, ICC	NSE, SP, somatostatin, CCK, glucagon, 13-norleucine-motiline, 5-hydroxytryptamine, neurotensin, BPP<, VIP, ACTH, GFA
Naik R [151]	1996	Neurogenic appendicopathy	Not stated	150	150	IHC	PTAH
Dhillon and Rode [152]	1983	Neurogenic appendicopathy	Not stated	50	24	IHC	NSE, serotonin, SP, VIP
Franke *et al.* [153]	2002	Neurogenic appendicopathy	Adults & children	282	96	H&E, IHC	S-100
Grebeldinger *et al.* [154]	2012	Neurogenic appendicopathy	Children	209	50	H&E, IHC	S-100
Sesia *et al.* [155]	2013	Neurogenic appendicopathy	Children	385	29	H&E, IHC	S-100
Di Sebastiano *et al.* [130]	1999	Neuroimmune appendicitis	Adults	47	15	IHC, computerised image analysis	VIP, SP, GAP-43
Bouchard *et al.* [134]	2001	Neuroimmune appendicitis	Children	19	9	IHC	VIP, SP, GAP-43
Xiong *et al.* [132]	2000	ENS & mast cells	Children	40	10	IHC, computerised image analysis	S-100, CNPase, Synaptophysin, NSE, mast cell tryptase
Amber *et al.* [135]	2010	ENS & mast cells	Adults	50	25	IHC, toluidine blue stain	S-100, NSE, mast cells
Nemeth *et al.* [127]	2003	Neuronal hypertrophy	Children	67	39	IHC, computerised image analysis	COX 1, COX-2, HLA II, iNOS
Wang *et al.* [129]	1996	Cytokine expression	Children	53	31	In-situ hybridisation	TNF-α, IL-2
Nemeth *et al.* [131]	2001	ENS & immune system	Children	82	31	IHC, whole-mount preparation	NADPH, nNOS
Vasei *et al.* [133]	2008	ECCs & SNCs	Not stated	98	26	IHC	Serotonin, chromogranin A, synaptophysin
Murphy *et al* [165]	2008	Cytokine expression	Children	22	2	Gene expression	IL-1, IL-2, IL-4, IL-5, IL-8, IL-12, IL-15 IL17, TNF, IFN-γ, p40

2.3.5 Clinical assessment and histologically "normal" appendicitis

2.3.5.1 Overview

The diagnosis of acute appendicitis is primarily clinical. Although, when the history and examination findings are typical it is easy to diagnose appendicitis, in some atypical cases the diagnosis can be very challenging. In the modern era, an increasing number of clinicians rely on additional testing and even on ultrasound, computed tomography or magnetic resonance imaging (MRI) to diagnose appendicitis and prevent delays in the management of the patient. A limited number of studies have described the presence of sign and symptoms as well as the levels of routine laboratory tests such as white cell count, neutrophil count, neutrophil to lymphocyte ratio and C-reactive protein (CRP) concentrations in patients with histologically "normal" appendices following a clinical diagnosis of acute appendicitis. However, the primary aim of most of those studies was to assess the trustworthiness of the clinical assessment in the diagnosis of acute appendicitis. Therefore, the research question was focused on the histologically inflamed samples and inevitably appendicectomies that resulted in histologically "normal" appendices were considered as "negative" or "unnecessary".

2.3.5.2 Signs and symptoms

Kalliakmanis *et al.* [166] designed a prospective study to assess the effectiveness of the initial diagnostic evaluation in patients with suspected appendicitis that was based on clinical findings. It was reported that 38.5% of patients with histologically "normal" appendices were found to exhibit signs of localised peritonism whereas 44.7% and 71.5% of patients suffering from uncomplicated and complicated acute

appendicitis respectively exhibited peritonism in the right iliac fossa. In the same study, 11.5% of patients with histologically "normal" appendices reported to have nausea/vomiting whereas the reported incidence of nausea/vomiting in patients with uncomplicated and complicated acute appendicitis was 36.8% and 31.3% respectively. With regards to anorexia, it was reported that 35.9% of patients with histologically "normal" appendices suffered from anorexia while incidence of anorexia in patients with uncomplicated and complicated acute appendicitis was 36.8% and 31.3% respectively. Finally, the authors reported that 26.9% of the patients with no evidence of inflammation on histology were pyrexial in the preoperative period compared with 36.8% and 54.2% of the patients with uncomplicated and complicated acute appendicitis respectively. Interestingly, the researchers reported that the intraoperative diagnosis of the appendiceal inflammation differed significantly from the histopathological diagnosis. Patients who according to the surgeon had a macroscopically normal appendix or evidence of mild appendicitis were in a more severe setting than thought to be. In a similar way, samples with histologically advanced appendicitis were thought to have milder inflammation. Similar results were published in two other studies [167, 168]. However, the latter have not included patients with histologically "normal" appendices.

2.3.5.3 Routine laboratory tests

In a study published by Shafi *et al.* [169], the authors attempted to assess the diagnostic and predictive value of preoperative WCC, neutrophil to lymphocyte ratio and CRP concentrations in patients with a clinical diagnosis of acute appendicitis who underwent appendicectomy. A total of 110 patients were included in the study of

whom 18 had a histologically "normal" appendix. The authors reported that 8 of the 18 patients with histologically "normal" appendices had abnormal WCC and 4 had abnormal CRP concentrations. Eleven of the 18 patients with histologically "normal" appendices were reported to have raised neutrophil count. Most of the patients with inflamed appendices (either uncomplicated or complicated appendicitis) had raised WCC, neutrophil count as well as CRP concentrations. However, the aim of the study was to assess the diagnostic and predictive value of routine laboratory tests and not the description of the levels between the different degrees of inflammation in the appendicitis spectrum and as a result statistical analysis to allow comparison of the results between the different groups of the study was not performed. Moreover, the study did not have a control group of normal appendices to allow comparison between the inflamed and the histologically "normal" samples.

A similar study to that of Shafi *et al* [169] was published by Gurleyik *et al.* [170]. The aim of the authors was to determine the accuracy of serum CRP concentrations in the diagnosis of acute appendicitis. The study included 108 patients of whom 18 had a subsequent histologically "normal" appendix. The authors reported that 2 of the 18 patients with histologically "normal" appendices demonstrated elevated concentrations of CRP. However, the CRP concentrations in those patients were not as high as in patients with inflamed appendices. On the other hand, the vast majority of patients with inflamed appendices on histopathologic examination demonstrated elevated CRP concentrations and those with complicated appendicitis were significantly higher compared to those with uncomplicated appendicitis.

In a very recent prospective study, Khan *et al.* [171] examined the role of neutrophil to lymphocyte ratio in assessing the severity of appendicitis. The study included 453 consecutive patients with a clinical diagnosis of acute appendicitis. However, 281 underwent appendicectomy as the rest were managed conservatively. Out of the 281 patients who had appendicectomy, 16 were excluded from the analysis as histopathological examination revealed a different pathology and 36 were found to have a histologically "normal" appendix. The CRP concentrations as well the neutrophil to lymphocyte ratio of those patients were elevated compared with normal values. Nevertheless, inflamed samples (either complicated or uncomplicated) demonstrated significantly elevated levels of CRP or neutrophil to lymphocyte ratio. However, as in the previously mentioned studies, the study by Khan *et al.* [171] this study did not aim to investigate symptomatic patients with histologically "normal" samples. Additionally, a large number of patients were treated conservatively as the clinical picture did not mandate appendicectomy. Thus, several patients from that group could have a histologically "normal" appendix altering the reported results significantly. Similar results were reported in a retrospective study but with a larger study population by Kahramanca *et al.* [172].

Two more studies associated a lower neutrophil to lymphocyte ratio with less severe inflammation in samples demonstrating features of appendicitis [167, 168]. It was reported that neutrophil to lymphocyte ration was significantly lower in appendices exhibiting minimal feature of inflammation on histological examination whereas complicated appendicitis samples demonstrated a noticeably high neutrophil to lymphocyte ratio. However, none of the studies examined the neutrophil to

lymphocyte ratio in patients with histologically "normal" samples or normal appendiceal tissue as control samples.

As a result, more studies are needed to focus primarily on the description of the signs and symptoms in combination with routine laboratory results of patients with a clinical diagnosis of acute appendicitis who were subsequently found to have histologically "normal" appendices. It would be useful in the understanding of the pathogenesis of appendicitis to compare the clinical data of these patients with patients with histologically proven appendicitis with varying degrees of inflammation.

2.3.5.4 Presence of faecolith

The presence of faecolith within the appendiceal lumen is considered as one of the principal causes of appendicitis [173]. It has also been assumed that complicated appendicitis has been associated with an obstructed lumen due to faecolith [174]. However, there have also been studies that questioned the theory of appendiceal faecoliths [175]. There is not enough experimental data to support or reject any of these theories and more importantly to associate the presence of faecolith with an altered inflammatory response in any of the different presentations of acute appendicitis. However, it has been shown that patients with faecoliths are less likely to respond to primary antibiotic therapy for acute appendicitis and usually need an appendicectomy [136].

Makaju *et al.* [176] investigated the theory that luminal obstruction due to a faecolith could be one of the cardinal causes of appendices and reported that in 518

appendices only 1.54% were found to feature faecoliths within their lumens. Histological examination demonstrated that early acute appendicitis was evident in 34.75% of the cases, acute suppurative appendicitis in 48.26% whereas gangrenous appendicitis was evident in 19.99% of the samples. The authors concluded that their findings did not support the concept that luminal obstruction secondary to faecolith could cause appendicitis [176]. The findings of Chandrasegaram *et al.* [177] were in agreement with those reported by Makaju *et al.* [176] as they reported that among 4670 appendices resected for faecoliths, worms, endometriosis or appendiceal tumours, 3.6% of the samples were found to have faecoliths and 39.5% were reported as histologically proven appendicitis. Therefore, their findings did not support the notion that luminal obstruction could be one of the causes of appendicitis.

In an attempt to explore this theory further, Singh and Mariadason [178] investigated the presence of faecolith in histologically "normal" and inflamed appendices from patients with a clinical diagnosis of acute appendicitis. It was reported that in 1014 emergency appendicectomy specimens, 28.6% of the histologically "normal" appendices, 29.9% of the paediatric and 13.7% of the adult histologically proven appendicitis specimens were found to have faecoliths. Moreover, it was reported that faecoliths were found in 39.4% and 14.6% of complicated and uncomplicated appendicitis samples respectively. However, according to the authors the appendicectomies that resulted in a histologically "normal" sample were considered as negative appendicectomies. Therefore, the authors concluded that the prevalence of faecoliths was too low to consider the presence of a faecolith as the most common cause of appendicitis [178].

Ramdass *et al.* [179] also investigated the association between the presence of a faecolith and inflamed (complicated or uncomplicated) appendices as well as histologically "normal" appendices resected from symptomatic patients. They reported that there was an association between the presence of faecoliths and uncomplicated appendicitis or histologically "normal" appendices but there was no association between the presence of faecoliths and complicated appendicitis. Therefore, the fact that the histologically "normal" appendices were considered to be truly healthy (negative appendicectomy), taken together with absence of association between the presence of faecolith and complicated appendicitis, led Ramdass *et al.* [179] to support the theory that faecoliths were mainly incidental findings and not a true cause of appendicitis.

In contrary to the two aforementioned studies, Alaedeen *et al.* [174] demonstrated that the presence of faecolith was associated with a higher perforation rate in children with acute appendicitis. A pre-operative CT scan was performed in 315 patients and faecoliths were detected in 30% of them. Histologically "normal" appendices that resulted in a negative appendicectomy were found in 12.2% of the patients. Four patients with histologically "normal" appendices had a faecolith present on the CT scan (0.03%). The authors also reported that an appendicolith was present on CT scan, intraoperative inspection or pathologic review in 31% of the patients. The appendix was found to be perforated in 57% of patients who had faecoliths and in 36% of patients without faecoliths. Their findings were consistent with previous studies [180-182]. Interestingly, patients with faecoliths had higher CRP concentrations on admission compared with those without faecolith but there was

no difference in the white cell count between the two groups of patients. The clinical observations of Alaedeen *et al.* were also in agreement with previously published studies [183, 184]. Moreover, the presence of faecolith was associated with a significantly shorter time interval between the onset of symptoms and presentation with perforation compared with patients without a faecolith. Thus, the authors concluded that the presence of a faecolith in a patient with clinical features of acute appendicitis significantly increases the risk of earlier perforation. Alaedeen *et al.* also hypothesised that erosion of the faecolith through the appendiceal wall could be another mechanism of perforation leading to early rupture. Therefore, abdominal contamination in patients with faecoliths would be more rapid as well as more extensive compared with contamination that resulted from perforation of appendices without faecolith [174].

The role of faecoliths as a cause of abdominal pain in patients with histologically "normal" appendices was assessed in a study conducted by Grimes *et al.* [185]. The researchers reported that a faecolith was found in 13% of patients with histologically "normal" appendices. 33% of patients with faecolith and 29% of patients without faecoliths reported pain in the right iliac fossa without any difference in the pre-operative duration of symptoms between the two groups. However, 52% of patients without faecolith reported to have recurrent episodes of pain after the operation while only 5% of patients with faecoliths experienced post-operative pain in the right iliac fossa. Therefore, the authors concluded that appendicular faecoliths could be associated with recurrent episodes of right iliac fossa pain.

Thus, it was apparent that the published data on the presence of a faecolith as a cause of appendicitis provided contradictory results. However, in several studies the histologically "normal" samples were considered as genuinely normal and the appendicectomy as negative. Therefore, more research is needed to examine how the presence of faecolith within the appendiceal lumen would affect the *in-situ* expression of several inflammatory markers as well as the magnitude of the inflammatory response before the increased intraluminal pressure due to faecolith can be considered as one of the causes of appendicitis. Furthermore, more studies are required to compare clinical parameters between patients with or without a faecolith in the appendiceal lumen.

2.4 Formulation of hypothesis and objectives

2.4.1 Overview

Appendicitis can present as an inflammatory spectrum. The conventional histology of the resected specimens can range from histologically "normal" when there is no microscopic evidence of inflammation, to acute inflammation with presence of neutrophils among other features of acute inflammation to gangrene and perforation that represent the most advanced stage of an inflammatory response. However, patients with histologically "normal" appendices surgically removed following a clinical diagnosis of appendicitis, may have subclinical inflammation that is not identified on conventional microscopy and resection of the appendix may prevent chronic or recurrent symptoms [127, 129, 131].

2.4.2 Hypothesis

This thesis tested the hypothesis that appendices which were resected from adult patients following a clinical diagnosis of acute appendicitis and reported as histologically "normal" on conventional histopathological examination, exhibited features of an inflammatory response evident at a subclinical level.

2.4.3 Objectives

The objectives of this study were:

- To describe the *in-situ* expression of TNF-α, IL-6, IL-2R and serotonin contents in samples of histologically "normal", acute uncomplicated and acute complicated appendicitis obtained from adult patients, with the use of immunohistochemistry.

- To describe differences in the signs (peritonism, nausea/vomiting, temperature), symptoms (duration, severity and previous episodes of RIF pain), laboratory investigations (WCC, NLR, CRP), intraoperative findings as well as other clinical parameters (presence of faecolith, pre- and post-operative stay) in patients with histologically "normal", acute uncomplicated and complicated appendicitis.

- To describe how the presence of previous episodes of right iliac fossa pain, localised peritonism and faecolith within the appendix influenced the expression of the studied inflammatory markers and clinical parameters in patients with histologically "normal", acute uncomplicated and complicated appendicitis.

- To explore whether the intraoperative appearance of histologically "normal" appendices reflected a difference in the expression of the studied inflammatory markers and the levels of the clinical parameters.

2.4.4 Need for further research

Following review of the available literature on appendicitis has become apparent that further research was needed as several aspects of its pathogenesis remain unclear. The vast majority of the previously published studies has focused on changes that occurred to the enteric nervous system of the resected appendices as a possible explanation of the signs and symptoms to suggest acute appendicitis in patients who were found to have histologically "normal" appendices. Some studies explored the concept of neurogenic appendicitis as they demonstrated either hyperplasia or hypertrophy of the nerve fibres in those patients with the use of immunohistochemistry whereas some earlier studies employed the role of microscopy (either conventional or electromicroscopy) to demonstrate close proximity of the nerve fibres to neuroendocrine cells. In order to further explore the pathogenesis of appendicitis and the symptomatology in patients with a subsequently histologically "normal" appendix, some authors investigated the interactions of the enteric nerve system with the immune system exploring the concept of neuroimmune appendicitis. Only three studies have focused entirely on the immune system of the appendix and the expression of inflammatory mediators that could explain the presentation of acute appendicitis in patients with histologically "normal" specimens [129, 131, 165]. However, since some of these studies were published, new evidence has emerged on the role of several cytokines and how they function depending on the type of inflammatory response. The function of TNF-α and IL-6 has been a characteristic example of this, as they could exhibit both inflammatory and anti-inflammatory actions. Therefore, the inflammatory response in acute appendicitis and more specifically in the

histologically "normal" appendices has not been adequately investigated and more studies are needed to explore the expression of inflammatory markers in the entire spectrum of appendicitis.

To the best of our knowledge, most of the already published studies have been conducted on a paediatric population and only two studies used appendix samples from adults [130, 135]. Both studies explored the concept of neuroimmune appendicitis and not subclinical immunohistological expression of inflammatory markers. In addition, some of the earlier studies have used a mixed population of both adults and children which could result in inaccurate data due to the significant variation of patient age [142, 148, 149, 153]. Moreover, the pathogenesis of appendicitis in children could be significantly different from that in adults. This became apparent from the study published by Nemeth *et al.* [131] who proved that the nerve fibre size was significantly different between paediatric samples and those originated from adults. Thus, further research is needed to investigate the pathogenesis of appendicitis in adults as well as the possible causes of symptoms in patients with histologically "normal" appendices.

Apart from the fact that most of the studies were conducted on children, the study population in most of them was relatively small given the prevalence of appendicitis in the general population. The study size in the published studies varied significantly from 19 [134] to 385 [155] patients. However, the number of patients with histologically "normal" appendices in the largest study that was published by Sesia *et al.* [155] was only 29. Most of the studies had a small number of histologically

"normal" appendices with only 3 to include more than 50 histologically "normal" samples [151, 153, 154]. In addition to small sample sizes, it was not possible to identify any study to examine specimens with evidence of gangrene or perforation in a separate group of samples from that with evidence of acute inflammation only. In most studies specimens with necrosis or perforation have either been excluded or included in the acute appendicitis group. The research team of the present study strongly believed that this group of patients was poorly investigated, and it could represent a different aspect of the disease and not only disease progression or different magnitude of the inflammatory response. As a result, not only bigger studies are needed but also studies to include samples from the entire spectrum of appendicitis which extends from histologically "normal" appendices, to acutely inflamed to samples with evidence of necrosis and/or perforation.

Finally, a small number of studies have combined the experimental data with clinical data that originated from the same population. However, to the best of our knowledge, there was no published study to combine the experimental data with data derived from patient's history and examination on the day of admission taken together with routine laboratory investigations as well as intraoperative findings. In the laparoscopic era, the diagnostic laparoscopy is frequently employed to help surgeons establish the correct diagnosis and initiate appropriate treatment. In the case of acute appendicitis, the surgeons are very often faced with appendices that appear grossly healthy and the dilemma of removing a macroscopically normal or a, as frequently described, "engorged" or "erythematous" appendix remains at surgeon's discretion. To the best of our knowledge, the expression of cytokines

between histologically "normal" appendices that looked inflamed and those that looked macroscopically normal during laparoscopy has not been investigated. Moreover, only one study examined the expression of cytokines in appendices with and without the presence of faecolith [165]. However, the group of histologically "normal" appendices included only two samples and both of them were found to have faecoliths within their lumens. On the other hand, none of the acutely inflamed samples had faecoliths. Therefore, the researchers were not able to reach any conclusions on whether the presence of faecoliths affected the expression of cytokines or not. Obstruction of the appendiceal lumen is considered one of the principal causes of acute appendicitis, and the presence of a faecolith is one of the most common causes luminal obstruction. Therefore, there is need for more studies on how the expression of various inflammatory mediators is affected by the presence of faecoliths.

The present study attempted to cover several gaps of the existing knowledge by examining the expression of key inflammatory markers in the entire spectrum of appendicitis ranging from histologically "normal" samples to acutely inflamed appendices without evidence of gangrene to complicated appendix specimens with evidence of gangrene or perforation in a large study with appendices derived from adult patients. It was also attempted to describe the experimental findings combined with clinical data from patients' clinical picture, routine laboratory tests as well as intraoperative findings in the context of the latest evidence on the function of the studied inflammatory markers.

2.4.5 Inflammatory markers examined in this thesis

2.4.5.1 Overview

This study has examined the expression of key inflammatory markers (serotonin, TNF-α, IL-6 and IL-2) in adult patients with clinical impression of appendicitis. These markers were specifically chosen due to their crucial role in the host immune response to antigenic stimuli as well as GI nociception. The expression of some of them has been previously assessed. However, most of the previous studies had a small sample size and were conducted on paediatric samples. In addition to this, new evidence has emerged for these markers since the previous studies were published and their expression needed to be further assessed in the context of the new evidence.

2.4.5.2 Serotonin

Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. 95% of the gastrointestinal serotonin can be found in the enterochromaffin cells (ECCs) with the rest contained within the ENS [186-188]. 5-HT synthesis from its precursor L-tryptophan (L-TPH) is mediated by tryptophan hydroxylase (TPH) in the ECCS [189]. ECCs are located between epithelial cells of the GI mucosa where they can sense the intraluminal milieu as they are equipped with specialised microvilli projecting into the lumen as well as electron-dense secretory granules and transporters that are located towards the apical parts of the cells [92, 190, 191]. Following ECCs stimulation, serotonin is predominantly released in the interstitial space as well as into the blood stream and the lumen, exhibiting various functions [92, 95, 192-194]. 5-HT can also

signal to CNS through activation of vagal afferent endings [92, 194] and its action is mediated through several receptors; 5-HT_1 to 5-HT_7 [195-200].

Several studies have highlighted the significant role of serotonin and ECCs in GI inflammation. In an older study it was demonstrated that both the number of ECCs and the 5-HT availability were reduced in patients with ulcerative colitis or IBS [201]. A more recent study by Magro *et al.* [202] also demonstrated decreased levels of serotonin in inflamed and non-inflamed colonic tissue from patients with either ulcerative colitis or Crohn's disease. However, Coates *et al.* [160, 201] showed a reduction in the number of serotonin producing ECCs in patients with severe ulcerative colitis but not in patients with mild ulcerative colitis. In a study that used computerised image analysis the area occupied by ECCs was increased in patients with IBD compared with the control group. The investigators attributed this increase to an increase in the number of ECCs as the mean individual cellular area of the endocrine cells types was the same in both the patient and control group [203]. Significant discrepancies in the number of ECCs as well as the level of 5-HT in intestinal tissue also described in experimental models of colitis [161, 204]. Moreover, Ghia *et al.* [205] investigated the role of endogenous 5-HT in intestinal inflammation using TPH deficient mice in a model of experimental colitis. It was reported that the reduced 5-HT synthesis resulted from the lack of TPH was not only associated with attenuation of the colonic inflammation but also with a reduction in expression of colonic IL-1β, IL-6 and TNF-α. Finally, in an in vitro study by Freire-Garabal *et al.* [206] the phagocytic activity of macrophages was significantly enhanced by serotonin. The findings of the aforementioned studies clearly show the crucial role of serotonin in

colonic inflammation as it is involved in the recruitment of inflammatory cells and cytokine expression.

The *in-situ* levels of serotonin have not been previously described in the whole spectrum of appendicitis. The ECC cells are strategically located between epithelial cells and have the ability to sense the intraluminal pressure. Therefore, due to the fact that luminal obstruction by faecolith is considered as one of the principal causes of appendicitis, it would be interesting to compare the serotonin levels in patients with and without evidence of faecolith within the appendiceal lumen.

2.4.5.3 Tumour necrosis factor alpha

TNF-α is one of the most important inflammatory mediators as it can exhibit a wide variety of functions in immunity, cell proliferation, differentiation, apoptosis as well as tissue repair and angiogenesis [207-213]. Although, it is mainly produced by macrophages and T-lymphocytes, a wide range of cells such as mast cells, NK cells, neutrophils, epithelial cells, endothelial, smooth muscle cells, fibroblasts and osteoclasts can also produce TNF-α. [212, 213].

Several studies have linked the expression of TNF-α in the intestinal mucosa with intestinal inflammation. TNF-α has been shown to activate intestinal microvascular endothelial cells and stimulate the expression of adhesion molecules as well as the production of TGF-β which is another potent pro-inflammatory cytokine [214]. Simpson *et al* [215] induced inflammatory bowel disease in mice, by transferring T cell-depleted wild-type bone marrow into syngeneic T cell-deficient (tge26) mice

(F1—tge26). This was accompanied by a prominent increase of TNF-α by colonic CD4+ and CD8+ TCRαβ T lymphocytes suggesting that the initiation of local inflammation in the colon of the transplanted tge26 mice was mediated by TNF-α. Neurath *et al.* [216] were among the first to describe the prominent overexpression of TNF-α in an animal model of colitis by showing that TNF-α transgenic mice developed fatal pancolitis following exposure to TNBS. Histological examination revealed substantial infiltrates of macrophages, granulocytes and T lymphocytes. The functional role of TNF-α in the experimental chronic colitis was established by demonstrating that TNF-α knockout mice did not develop mucosal inflammation following administration of TNBS. Moreover, treatment with anti-TNF-α antibodies ameliorated both clinical and histological signs of colonic inflammation [217]. The first observation of overexpression of TNF-α in humans with inflammatory bowel disease was reported by MacDonald *et al.* [218] who demonstrated with the use of spot ELISA that all patients with Crohn's disease, demonstrated increased levels of TNF-α.

Although TNF-α has some well documented pro-inflammatory properties, there is strong evidence to suggest that TNF-α can also exhibit anti-inflammatory activities. With the use of an experimental model of colitis, Naito *et al*. [219] demonstrated that colonic inflammation was considerably exaggerated in genetically TNF-α deficient (-/-) mice. Neutrophil infiltration was also shown to be markedly enhanced in the TNF-α deficient mice and the survival rate of TNF-α deficient mice was significantly lower compared with TNF-α +/+ mice. These results suggested that TNF-α has an immunosuppressive role by downregulating the magnitude and the duration of the colonic inflammation. In a similar way, Kojouharoff *et al.* [220] demonstrated that

neutralisation of TNF-α led to exacerbation of acute colitis, demonstrating a protective role of TNF-α in the acute inflammation. However, in a model of chronic inflammation, neutralisation of TNF-α promoted epithelial damage and immune cell infiltrate indicating that TNF-α has the ability to perpetuate a chronic inflammatory status. TNF-α has also been implicated in the production of glucocorticoids by intestinal epithelial cells [221]. Extra-adrenal production of glucocorticoids by the intestinal epithelial cells has been shown to play a vital role in the maintenance of the colonic epithelial cell barrier by stimulating the production of adhesion molecules and hence, promoting tight junction formation between the epithelial cells. It is known that excessive apoptosis of epithelial cells in patients with inflammatory bowel disease leads to disruption of the epithelial barrier and impaired synthesis of glucocorticoids. This subsequently leads to entry of proinflammatory factors such as bacterial LPS into the colonic wall exacerbating the disease even further. On the other hand, systemic glucocorticoids have been shown to inhibit the pro-inflammatory action of TNF-α by downregulating the expression of the TNF-α gene [222].

To the best of our knowledge the expression of TNF-α has not been studied in samples with appendicitis that originated from adult patients. The previously published studies using paediatric appendices have provided conflicting results regarding the gene expression of TNF-α [129, 165]. However, both studies had a small number of samples. It would be interesting to document the number as well as the location of positively stained cells for TNF-α with the use of immunohistochemistry. Moreover, since the first study on the expression of TNF-α in appendicitis was published, new evidence has surfaced on the ability of TNF-α to function as pro- and

anti-inflammatory mediator. Therefore, this work sought to explore the differences in TNF-α expression in appendix specimens following a clinical diagnosis of acute appendicitis but with substantial differences in the clinical presentation, intraoperative findings, routine laboratory results and most importantly with different degrees of inflammatory response in the context of new evidence.

2.4.5.4 Interleukin 6

Interleukin 6 (IL-6) is another important inflammatory mediator that exhibits a plethora of functions and can act as both proinflammatory and anti-inflammatory cytokine [223]. IL-6 is mainly produced by macrophages, B- and T-lymphocytes, epithelial and endothelial cells as well as smooth muscle cells, fibroblasts and osteoblasts [224, 225].

IL-6 has been shown to exhibit pro-inflammatory actions with significant systemic effects. The first evidence to associate IL-6 with the expression of acute phase proteins originated from studies in human hepatocytes. It was demonstrated that IL-6 was able to induce C-reactive protein and amyloid A synthesis [226]. Moreover, IL-6 was able to induce expression of the full spectrum of acute phase proteins, whereas, IL-1 and TNF-α had only a moderate effect on acute phase proteins suggesting that IL-6 has a key pro-inflammatory action [224]. It has also been shown that IL-6 can act directly on B-lymphocytes by inducing production of immunoglobulins M, G and A. IL-6 has been demonstrated to play a crucial role in the activation and proliferation of antigen-receptor-dependent T lymphocytes as well as be a potent pyrogen [224].

Apart from well described pro-inflammatory properties, IL-6 can also exhibit a wide variety of anti-inflammatory actions. It has been shown that absence of IL-6 resulted in a significantly higher circulating level of pro-inflammatory cytokines during endotoxemia such as MIP-2, IFN-γ and TNF-α. Each of these cytokines have been implicated in the elicitation of toxic syndromes in endotoxemia. Moreover, the anti-inflammatory action of IL-6 has been further supported by the observation that IL-6 -/- mice suffered from increased mortality when given a high dose of endotoxin. Moreover, it has been shown that administration of recombinant IL-6 was able to induce the expression of anti-inflammatory molecules IL-1 receptor antagonist and soluble TNF-α receptor and to inhibit TNF-α in experimental endotoxemia. It has also been shown that IL-6 was capable of inhibiting neutrophilia and stimulate the release of anti-inflammatory molecules such as anti-protease inhibitors [223]. This was further supported by the finding that IL-6 was able to restrict recruitment of neutrophils promoting their replacement by mononuclear cells steering the inflammatory response towards chronicity [227-229]. Therefore, it has been clearly documented that IL-6 is also a potent anti-inflammatory mediator.

Although, IL-6 is one of the most potent pro-inflammatory mediators, its expression has never been studied in appendicitis. In this study there was an opportunity to compare the levels of expression of this key pro-inflammatory mediator in the whole spectrum of appendicitis from histologically "normal" appendices to specimens with areas of gangrene or perforation. However, as IL-6 has been shown to inhibit neutrophilia and direct the inflammatory process towards chronicity, it would be of paramount importance to assess IL-6 expression in patients with recurrent episodes

of right iliac fossa pain and histologically "normal" appendices and compare it with that in samples with evidence of acute inflammation without prior episodes of right iliac fossa pain that could suggest the presence of a chronic inflammatory response.

2.4.5.5 Interleukin 2

Among the different cytokines, IL-2 is one the most important ones in the generation and regulation of immune responses [230, 231]. IL-2 is mainly expressed by activated CD4+ and CD8+ T-lymphocytes. It is also produced by activated dendritic cells, natural killer as well as natural killer T-lymphocytes and its actions are mediated by the IL-2 receptor (IL-2R) [232-237].

Among the most important pro-inflammatory action of IL-2 is its ability to promote proliferation of CD4+ and CD8+ T-lymphocytes as well as stimulate the expression of NK-derived cytokines such as TNF-α and IFN-γ. IL-2 has also been shown to upregulate expression of heavy and light chain genes in IgM producing B-lymphocytes. As in T-lymphocytes, IL-2 further increases expression of IL-2R in B-lymphocytes, thus enhancing their responsiveness to IL-2 [238]. Finally, Dignass and Podolsky [231] demonstrated that IL-2 could act as a link between the intestinal epithelial cells and the lamina propria and thus intergrade the epithelial surface to the intestinal immune system. It was shown that the rat intestinal epithelial cells exhibited IL-2 receptors in their surface responsible for epithelial cell proliferation and migration [231].

IL-2 also plays an important role in downregulating immune responses. Previous studies have demonstrated that genetically modified mice with IL-2 gene knock-out

(IL-2 -/-) spontaneously developed bowel inflammation [239, 240]. Sadlack *et al.* [47] demonstrated that IL-2 -/- mice were normal at birth but they could die between 5-9 weeks of an unrecognised disease which was charecterised by splenomegaly, lymphadenopathy and haemolytic anaemia. Nevertheless, the mice that survived beyond 9 weeks developed colonic inflammation that resembled human ulcerative colitis. Further characterisation of IL-2 gene knock out mice revealed that these mice could develop colitis characterised by increased IFN-γ, TNF-α and IL-1 in combination with decreased expression of IL-4 and IL-10 [241, 242]. However, between the age of 10-18 weeks, the wild type demonstrated an increase in the endocrine cells myenteric nerves expressing VIP and Substance P, while no such increase was observed in healthy heterozygous or diseased IL-2 -/- mice. These results demonstrated the vital role of IL-2 in the interactions between the neuroendocrine and immune system of the GI tract. In a recent study, Nikiforou *et al.* [243] showed that intravenous administration of IL-2 can protect the fetal gut against inflammation and mucosal injury in the course of chorioamnionitis. The protective effect of IL-2 administration could be explained by different ways. IL-2 could protect foetal intestine against inflammation by preventing IL-17 induction as IL-17 mRNA levels were found to be reduced 2 days after IL-2 treatment. IL-17 is known to promote neutrophil recruitment and colonic inflammation [244-246]. Another possible explanation could be the inhibition of IL-6, as IL-6 has been implicated in the epithelial tight junctional loss and mucosal injury [247]. It could also be the direct action of IL-2 on the epithelial cells as IL-2 preserved the epithelial integrity by promoting epithelial cell restitution and cell proliferation [231]. Therefore, it was clear that IL-2 played a crucial role in the regulation of immune responses.

Despite the significant amount of knowledge on the actions of IL-2 in colonic inflammation, its role in appendicitis has not been studied enough as only one study has assessed its expression in a paediatric population [129]. As mentioned earlier, IL-2 is expressed by activated CD4+ and CD8+ T-lymphocytes and therefore increased expression of IL-2 would indicate an activated immune system. This thesis attempted to assess whether the immune system of patients with histologically "normal" appendices was activated or not. Thus, the *in-situ* level of IL-2 would indicate whether the histologically "normal" samples were truly normal or not. Moreover, increased expression of IL-2 could indirectly imply that the expression of IL-17 could be low and therefore the inflammatory response of appendicitis would not exhibit features of Th17 immune response. Finally, since the publication of Wang *et al.* [129], new evidence has highlight the role of IL-2 as an anti-inflammatory cytokine and this work endeavoured to describe IL-2 expression in this context using a large population of adults.

CHAPTER 3: Materials and methods

3.1 Overview

In-situ expression of two cytokines (IL-6, TNF-α), a cytokine receptor (IL-2R) and one neuropeptide (serotonin) was studied using immunohistochemistry on paraffin-embedded samples that were retrospectively collected from the archive of the Department of Histopathology at Queen's Medical Centre, Nottingham University Hospitals NHS Trust in Nottingham, UK. Clinical data relevant to the underlying pathology and associated to the reason for admission to hospital were collected from the patient notes as well as the electronic patient database of Nottingham University Hospitals NHS Trust.

The study was given approval by Health Research Authority. Reference Number: 18/HRA/0292. The study Registration number was: 233124/1129322/37/873.

3.2 Specimens and clinical data

3.2.1 Inclusion criteria

The appendix specimens included in this study were obtained from patients who underwent emergency appendicectomy between September 2013 and May 2011 in a consecutive order until the required number of patients for each group was achieved. Queen's Medical Centre is the tertiary hospital of the region of East Midlands. The samples derived from adult patients of all ages, genders, ethnicities and socio-economic groups following a clinical impression of acute appendicitis. As a result, the study population was a true representation of the adult population of City of Nottingham and subsequently of the challenges and dilemmas that general surgeons face on a day to day basis. The control group was consisted of specimens from patients who underwent elective abdominal surgery and the appendix was removed as part of the resected colon.

The appendix specimens obtained at surgery were fixed in formalin and divided into three sections according to the protocol of the Department of Histopathology (2 longitudinal sections with one originating from the tip and the other from the resection margin and one transverse section form the middle of the appendix). Following retrieval of the slides from the archive of the Department of Histopathology, they were re-examined by a senior Histopathology consultant (Dr. A. M. Zaitoun) and were given a diagnosis which was based on the severity of inflammation (histologically "normal", uncomplicated acutely inflamed, complicated acutely inflamed). Dr A. M. Zaitoun was blinded to the initial diagnosis as stated on the official Histology report following the routine histopathological examination of

the sample post-operatively. In order for the specimens to be included in the study, the diagnosis made by Dr A. M. Zaitoun had to match with the initial diagnosis on the Histology report. The appendix specimens for the control group were also re-examined by Dr A. M. Zaitoun and the diagnosis of being normal had to match the initial diagnosis on the Histology report for them to be included in the study. Therefore, each sample that has been included in the study was examined by two Histopathology consultants who independently agreed on the underlying pathology.

3.2.2 Exclusion criteria

Several factors were considered for the exclusion criteria and were divided into patient factors and specimen factors. With regards to patient factors, appendix specimens obtained following interval appendicectomy were excluded from the study as patients were not admitted as an emergency and therefore there was not a diagnosis of acute appendicitis at the time the appendix was obtained. Samples that demonstrated features of sub-serosal inflammation with a healthy mucosa were not included in the study as this finding could indicate the presence of an inflammatory process arising elsewhere in the abdomen. Characteristic example of this, is pelvic inflammatory disease or other pelvic pathology in patients who presented with right iliac fossa pain, underwent a diagnostic laparoscopy and the decision was made for the appendix to be removed. Patients who were admitted months or even years after the appendicectomy and were diagnosed with inflammatory bowel disease were also excluded from the study as the right iliac fossa pain and the subsequent diagnosis of acute appendicitis could potentially indicate the first presentation of inflammatory bowel disease. Finally, as mentioned in the inclusion criteria section, patients whose

diagnosis by a second Histopathology consultant did not match the initial diagnosis on the Histology report post-operatively were excluded from the study.

As far as the control group was concerned, patients that were identified to suffer from inflammatory bowel disease following review of the clinical data were excluded from the study as that could affect the expression of the examined inflammatory markers within the appendix. Moreover, patients with obstructing tumours anywhere in the colon were also excluded as it was thought that increased intraluminal pressure could also affect the expression of different inflammatory markers even though there was no microscopic evidence of inflammation on conventional histology. Patients with caecal tumours as indicated on the histology report were excluded from the study due to the close proximity of the tumour to appendix and the possible effect that this proximity could have on the expression of the studied markers.

Specimens with extensive necrosis were excluded from the study as that would compromise the quality of immunohistochemistry and would not allow examination of several different microscopy fields. Appendix samples with significant amount of calcification or evidence of poor tissue fixation were also excluded as that would affect the quality as well as the thickness of the sections and as consequence the immunostaining would not be consistent.

3.2.3 Pathological characteristics and clinical data

A computerised search was conducted in the database of the Department of Histopathology at Nottingham University Hospitals NHS Trust to retrospectively identify consecutive histology reports involving appendix specimens. The histology reports as well as patient notes were reviewed in order to make sure that the appendicectomy was performed following a clinical diagnosis of acute appendicitis. This was followed by retrieval of consecutive histology slides that were re-examined by a senior Histopathology consultant (Dr. A. M. Zaitoun) and allocated into four groups. Group I included patients with histologically confirmed uncomplicated acute appendicitis (n=120). The H&E stained sections of this group could demonstrate signs of acute inflammation such as vasodilation, oedema, infiltration of the different layers of the appendix with polymorphonuclear leucocytes, ulcers of the epithelium as well as local abscesses and fibrinous exudates on the serosa of the appendix. Group II included patients with histologically confirmed complicated appendicitis (n=118). The appendix specimens of this group demonstrated features of acute inflammation with additional evidence of gangrene and/or perforation on conventional microscopy. Group III included patients that histological examination of their appendix did not show any of the signs of acute inflammation (n=104). In the specimens of this group the epithelial layer was intact and the histological architecture of the mucosa, submucosa and *muscularis propria* had normal appearance. The control group (Group IV) was consisted of appendices removed as part of the resected colon for pathology other than appendicitis (n=106). The specimens of this group had no evidence of inflammation with an intact epithelial layer and the histological architecture of all the layers was normal.

Clinical data on patient's history and examination findings was retrospectively collected from the surgical clerking proforma of Nottingham University Hospitals NHS Trust as it was documented on the day of admission. Several members of the surgical team were able to document patient consultations and examinations that took place. The on-call surgical team staffing the Surgical Admission at the Queen's Medical Centre consisted of a Foundation Year 1 doctor, one Core Surgical Trainee/Senior House Officer, two Higher Specialty Trainees/Registrars and a Surgical Consultant. The level of training and experience of the different members of the surgical team varied significantly with the Foundation Year 1 doctor being the least experienced and the surgical consultant the most experienced member of the team. The data for this work derived from entries that were made by the most senior members of the team on the admission proforma. This data involved the duration of symptoms that made the patient to seek medical advice as it was stated by the patient on admission to the Surgical Admission Unit. Data regarding the presence of previous episodes of right iliac fossa pain from patient's history as it was recorded on the surgical clerking proforma was also collected. The presence or absence of nausea and/or vomiting was recorded regardless the frequency of vomiting. The presence or absence of localised peritonism was also recorded with localised peritonism defined as the presence of abdominal guarding or rebound tenderness over the McBurney's point. With the data derived from patient notes, the length of pre- and post-operative stay for patients with acute uncomplicated appendicitis, complicated appendicitis and histologically "normal" appendices was calculated.

Data on the severity of right iliac fossa pain was collected from the admission documentation that the nursing staff had to complete as soon as the patient arrived on the Surgical Admission Unit. Each patient was asked to rate his or her pain on a scale from 0 to 10. Zero represented the absence of pain and 10 was the worst pain that the patient had ever felt.

With regards to the intraoperative impression of the operating surgeon whether the subsequently histologically "normal" appendices looked inflamed or not, data was collected from the operative record as it was documented by the surgical consultant or registrar who performed the appendicectomy. Apart from clear documentation that the appendix looked inflamed, expressions such as injected appendix, engorged appendix and hyper-vascular appendix were also classified as inflamed appendix.

Data was collected on the presence of faecolith for the groups of patients with acute appendicitis, complicated appendicitis and histologically "normal" appendices. The reports of the imaging tests that patients underwent on admission (abdominal USS or CT scan) were reviewed for the presence of faecolith within the appendix. The operative record was also reviewed for evidence of faecal material when the appendix was divided as well as the histology report for evidence of faecal material within the appendicular lumen during preparation of the specimens for histological examination. By collecting data on the presence of faecolith from all the available modalities that could identify faecoliths the chances of missing appendix specimens with faecoliths were minimised.

Regarding the results of routine laboratory investigations that are commonly used to guide the surgical team, data on white cell count, neutrophil count, neutrophil to lymphocyte ratio and C-reactive protein concentrations was collected. All these markers are used as indicators of the severity of the inflammatory process. The collected data on these markers was originated from the first blood sample that was obtained from the patient on the day of admission. All the blood samples were processed by the Biochemistry laboratory at the Queen's Medical Centre and the results were uploaded to the hospital's electronic database called Notis. Data on these markers for the patients of the control group were not collected as most of these patients had their last blood test several days or weeks prior to the elective abdominal surgery during the pre-operative assessment and not on the day the appendix was obtained.

Finally, data was obtained on the body temperature of the patients with acute uncomplicated appendicitis, acute complicated appendicitis and histologically "normal" appendices. First body temperature was defined as the measurement that was recorded by either the General Practitioner who referred the patient to the Surgical Admission Unit or the team in Accident and Emergency where the patient presented with abdominal pain. The first recorded body temperature was considered to be an important indicator of the inflammatory process as it could influence the decision of the General Practitioner or Accident and Emergency team to refer the patient to the surgical team. The highest body temperature in the pre-operative period was also recorded while the patient was under the care of the surgical team. This measurement was considered to be a marker of severity of the inflammatory

factor and an important factor that could possibly influence the decision made by the surgical team to proceed with appendicectomy.

The allocation of specimens into 4 groups allowed for the expression of the selected markers to be studied in the entire spectrum of appendicitis, from histologically "normal" appendices obtained from symptomatic patients, to acutely inflamed samples, to complicated appendicitis with evidence of gangrene or perforation. It is widely acceptable that the group of patients with gangrenous or perforated appendicitis has not been adequately studied. This could be due to the technical difficulties in using tissues with necrosis or because gangrenous appendicitis has always been considered as progression of the inflammatory status and therefore did not attract much interest. In some of the previously published studies, samples with evidence of necrosis were examined in the same group with appendices that exhibited features of acute inflammation only. Thus, the published data might not be representative of the underlying pathology. The allocation of the specimens into four groups samples also facilitated the comparison of the different clinical parameters between patients with different aspects of the appendicitis disease. To the best of our knowledge there has not been a previously published study to investigate the expression of cytokines within the appendix tissue in the entire spectrum of appendicitis along with data from clinical findings, routine laboratory investigations and intraoperative findings.

3.3 Immunohistochemistry

3.3.1 Overview

Immunohistochemistry (IHC) is a robust tool for gaining more information on tissue specimens compared with the conventional histological examination. IHC can be used not only to study the expression of cellular markers defining cellular/tissue phenotypes but also the location of proteins/molecules in tissue samples. The added information can be used for diagnostic, prognostic and predictive purposes related to a disease status and normal biology. Therefore, the application of enzyme-labelled antibodies to study formalin-fixed tissue samples provides an additional parameter in the assessment of inflammation biology.

Immunohistochemistry was chosen as a method to assess the *in-situ* expression of the studied inflammatory markers due to its inherent advantages. As it examines protein expression, IHC can provide information on the final product of gene expression. Increased gene expression does not always translate to increased protein levels as the RNA interference system is able to silence gene expression at the mRNA level. Immunohistochemistry has also the advantage of demonstrating the location of the protein within the tissue architecture, it is easily reproducible and more economical compared to gene analysis especially in studies with a large sample size. Immunohistochemistry has been used successfully not only for research purposes but also for diagnostic, prognostic as well as predictive purposes in patients with malignancies. Several studies on colorectal cancer have compared immunohistochemistry with PCR and have shown that the coincidence rate of the two methods for detecting microsatellite states was 91.92% [248-251].

3.3.2 Antibody class and structure

Antibodies entail a group of proteins called immunoglobulins (Igs) which are produced by B lymphocytes following stimulation by antigens and which react specifically with those antigens. They are formed in the blood as well as the peripheral tissues of normal individuals. Ig comprises five classes (IgG, IgA, IgM, IgD and IgE). Each antibody has two identical heavy chains and light chains. The antigenic and structural properties of the heavy chains vary significantly and define the class of Ig. The most frequently used antibody in IHC is the IgG. IgG consists of two monovalent antigen-binding fragments (Fab) and one crystalline fragment (Fc). Additionally, IgG particles can be split into variables and constant domains. The variable domains of heavy and light chains create the antigen-binding site (paratope) which specifically recognises the antigenic determinant (epitope) of an antigen [252]. Monoclonal antibodies are produced by a single B cell clone and form a homogenous population of Ig that can target a single epitope. Every monoclonal antibody has unique specificity and affinity. On the other hand, polyclonal antibodies are heterogenous as they consist of a mixture of antibodies directed against several epitopes of the same antigen. Polyclonal antibodies are harvested from the blood of animal which have been immunised with a specific antigen. On one hand, their ability to target several epitopes on an antigen makes them more reliable compared with the monoclonal antibodies when they are used on routinely processed samples. On the other hand, being able to recognise multiple epitopes, increases the chance for cross reactivity with other antigens or proteins. Moreover, a lot to lot inconsistency is also a disadvantage of polyclonal antibodies [253].

3.3.3 Antibody affinity and titre

Functional affinity of an antibody is defined as the total time required to saturate all available antibody binding sites with antigens (equilibrium). Therefore, antibodies with low affinity will require longer time to reach the maximal staining intensity. On the other hand, intrinsic affinity of an antibody refers to the concentration of an antigen to saturate the antibody. Therefore, an antibody with higher intrinsic affinity will need lower concentration of an antigen to reach equilibrium. Affinity can also refer to the binding strength between the antigen and the antibody (immune complex). In IHC, the antigen-antibody binding is reversible and may dissociate during the phase of washing. The dissociation of an immune complex can be reduced by lowering the temperature.

The ideal antibody titre is the highest dilution which can achieve a maximum of specific staining intensity with the least non-specific background staining under the predetermined conditions. The ideal dilution is also determined by antibody affinity. In a given dilution and incubation period, a low affinity antibody will result in weaker staining intensity compared with an antibody with high affinity.

3.3.4 Incubation period

The incubation time, temperature as well as antibody titre and affinity are interdependent factors as a change in one will influence the others. Incubation time can vary from 10 minutes to 24 hours. In general, the use of higher antibody titre will result in less incubation time. The most widely used incubation time is 10-30 minutes as it is adequate for antibodies with high affinity and concentration to be saturated

with their antigens. However, a 24-hour incubation time offers economic benefits as the amount of the antibody needed is significantly reduced.

IHC is most commonly performed at room temperature (25 °C). The equilibrium of antigen-antibody can be reached faster at 37 °C. However, while increasing temperature allows the use of a shortened incubation time, increased background staining may appear. A temperature of 4 °C is commonly used for 24-hour or overnight incubation. A humidity room is necessary to prevent drying/evaporation of the tissue sections [254].

3.3.5 De-masking of antigens

The 3-dimensional structure of proteins within the tissue is altered during formalin fixation of the specimen. Therefore, antigenic epitopes can be masked or even destroyed resulting in inability to react with antibodies. De-masking of antigen or epitope retrieval is the process that can reverse this by the protein cross-links, which were formed during the fixation, in order to expose the antigen epitopes. Antigen de-masking can be done by heating the sample for a certain period of time [heat-induced epitope retrieval (HIER)] or using enzymatic digestion. Despite the fact that many epitope retrievals have been described, the most commonly used is heating the specimens in a microwave oven with citrate buffer pH 6.0. Nonetheless, the optimal protocol for each antigen/antibody interaction needs to be determined [253, 255].

3.3.6 Immunoenzymatic staining with enzyme substrate reactions

In immunoenzymatic staining methods, such as IHC, colourless substrates are converted into coloured end products with the use of enzymes. Several factors such as incubation time, temperature, pH, concentration and light can affect the catalytic reaction of those enzymes. Among the most commonly used enzymes are the Horseradish peroxidase (HRP) and the calf intestine alkaline phosphatase (AP). Substrates become coloured following oxidation by HRP. The colour of the end-product depends on the chromogen used. Diaminobenzidine (DAB) has been used in this study, as it is one of the most commonly used in IHC and the end-product is brown in colour. It is highly stable in alcohol solvents used in IHC. Haematoxylin and non-aqueous DPX (dibutyl phthalate) mounting medium can also be used with DAB. Moreover, it was endeavoured to control all the factors that can affect the catalytic reactions during each IHC run. The incubation time of each substrates was strictly kept with the use of a timer. The concentration and the pH of each substrate was either prepared according to the information sheet supplied by the manufacturer or as it was already supplied by the manufacture (e.g. Novolink™ polymer detection system, Leica® RE7280-K). The experiments were conducted in a certified laboratory of the University of Nottingham that provided stable conditions such as temperature and light [254].

3.3.7 Secondary antibody: Labelling reagents and amplifications

Several amplification methods have been described in order for even small amounts of antigen to be detected by a high sensitivity test. The intensity of the staining can be significantly increased by using a conjugated/labelled secondary antibody.

Therefore, even a faint but specific signal can be amplified without compromising the specificity [256]. Streptavidin and avidin molecules have four binding sites with strong affinities for the vitamin biotin. HRP is easily conjugated to biotin. Therefore, a single antibody can be associated with several peroxidase molecules and a larger enzyme to primary antibody ratio can increase the sensitivity of the of the antigen detection [257]. However, the existence of endogenous biotin in the tissue can lead background staining and blocking the endogenous biotin is not very effective. The polymer based IHC technique that has been used in this study does not depend on endogenous biotin as it uses a polymer dextran backbone in which several secondary antibodies and peroxidase enzymes can be conjugated. The secondary antibody attached to this polymer comprises of both anti-rabbit and anti-mouse Ig molecules. Hence, it can be used to detect primary antibodies of either rabbit or mouse origin [258].

3.3.8 IHC standardisation

Although, IHC has been shown to be a very effective technique in demonstrating related biomarkers in diagnosis and classification of tumours, concerns have been expressed regarding the validation of the reagents, the reproducibility of the staining methods and the interpretation of the results. Despite the fact that several attempts have been made to standardise IHC techniques, standardisation remains largely a critical issue. Several intrinsic factors such as variable conditions in tissue processing and fixation that can affect the preservation of antigens remain uncontrolled [259]. Fixation of the tissue prevents proteins in the specimen from degradation and modification as well as preserves the location of the antigens whether they are found

in the nucleus, in the cytoplasm or in the membrane. On the other hand, the use of formalin has a negative impact on the antigenicity of the tissue. Moreover, as necrotic degradation of the tissue starts immediately following devascularisation of the tissue, the time to fixation is vital. Inequivalent IHC staining can be obtained among formalin-fixed paraffin-embedded (FFPE) tissue samples with different periods of time between tissue fixation and staining [260]. The utilisation of validated reagents, optimised conditions and protocols, positive and negative controls is vital to ensure the reproducibility of a test with high sensitivity and specificity.

3.3.8.1 Primary antibody validation and optimisation

The selection primary antibodies with the required specificity in detecting the target antigen is based on the data supplied by the manufacturers. Even though these antibodies have been tested for specificity by reliable manufacturers, it is of paramount importance to confirm functional specificity by using appropriate positive and negative control specimens. The optimal staining intensity in combination with the lowest level of non-specific background staining can be achieved by adjusting the concentration of the primary antibody for each staining condition with the use of a particular secondary labelling system. The optimal working concentration can be found by experimenting with serial titrations. The concentration of primary monoclonal antibody can be accurately determined as μg/ml but it can only be estimated for polyclonal antibodies. Therefore, a dilution fraction e.g. 1:50 or 1:100 is commonly used instead of an accurate concentration.

3.3.8.2 Positive and negative controls

Both positive and negative controls are tissue samples that have been fixed and processed in the same way to the test specimens. Positive controls are known to have the target antigen and should demonstrate a range of staining intensity. This will help greatly in selecting the optimal working dilution. Positive control samples that contain high levels of the target antigen can lead to the selection of a working dilution that will fail to detect low levels of target antigen in the test specimens. The use of negative controls that are identified by the absence of staining incorporates two concepts [261, 262].

3.3.8.3 False positive and negative errors

The presence of endogenous peroxidase activity has been demonstrated in red blood cells (haemoglobin), muscle fibres (myoglobin), inflammatory cells such as granulocytes and monocytes (cytochrome) as well as liver and kidney (catalases). It can give rise to false positive staining when HRP-based detection methods are used and therefore, suppression of its activity is essential prior to the addition of primary antibody during the during the staining process. This can be achieved by incubation of the tissue sample in 3% H_2O_2 for 5-10 minutes.

Both primary antibodies and detection systems are protein-based reagents that can react with non-specific binding sites in the tissue sample. This will result in false positive as well as background staining. The use of a protein blocking reagent is essential in order to reduce non-specific protein-protein interactions between IHC reagents and tissues.

False negative staining can arise following inappropriate tissue handling, fixation, processing or antigen retrieval due to loss of antigenicity in the specimen. Moreover, false negative staining can ensue when steps of the staining protocol are omitted accidentally [263].

3.3.8.4 Computer-aided scoring

The assessment of the immunostaining has been conducted mainly by human visual scoring. The scoring parameters are generally predetermined by quantitative or semi-quantitative cut-off values, resulting in categorical values for statistical analysis. The IHC assessment can be very time-consuming when carried out by human interpretation. Moreover, pathological examination of tissue samples remains a subjective process as the intensity of the observed staining and the subsequent scoring are prone to visual bias. A solution to these issues is the utilisation of computer-assisted scoring with scanning devices and image analysis software. The computer-assisted scoring is not subjected to confounding factors such as fatigue and ambient light, and also minimises the issue of inter-observer discrepancies [264]. It has been demonstrated that computer-aided scoring is a reliable and more time-effective alternative to human visual scoring. However, it does not provide an analytical advantage [265-267].

The main disadvantages of the computer-assisted scoring consist of the inability to classify tissue type and distinguish membrane, cytoplasmic and nuclear staining as well as the inability to differentiate tumour from non-tumour tissue or necrotic from non-necrotic tissue. Moreover, it has been shown that more time is needed for

subsequent analysis using automated IHC scoring [265]. Therefore, the use of manual IHC assessment, as described in the section 3.4.4 Assessment of immunohistochemical, staining has been selected as the preferred method of scoring in this study.

3.4 Methods

3.4.1 Overview

The *in-situ* expression of inflammatory markers IL-6, TNF-α, IL-2R and serotonin was studied using immunohistochemistry on formalin-fixed, paraffin-embedded samples. The archived tissue blocks were melted, and two tissue specimens were re-embedded into a new tissue blocks (Figure 3.1) using the Thermo Scientific® HistoStar™ embedding module (Figure 3.2). All specimens were sectioned using the LEICA® RM2125 Microtome (Figure 3.3) and mounted on Surgipath™ X-tra™ Adhesive Leica® slides. Subsequently, the sections were immunostained according to an established protocol (Appendix-1 IHC protocol) using 4 antibodies to determine expression of inflammatory markers. The stained slides were scored on a Zeiss® Axioskop 2 microscope (Figure 3.4). Photographs were taken using a Nikon® Eclipse 80i microscope with a Nikon® DS-L3 digital camera system (Figure 3.5).

Figure 3.1: Archived tissue blocks (right) and new tissue blocks following re-embedding (left).

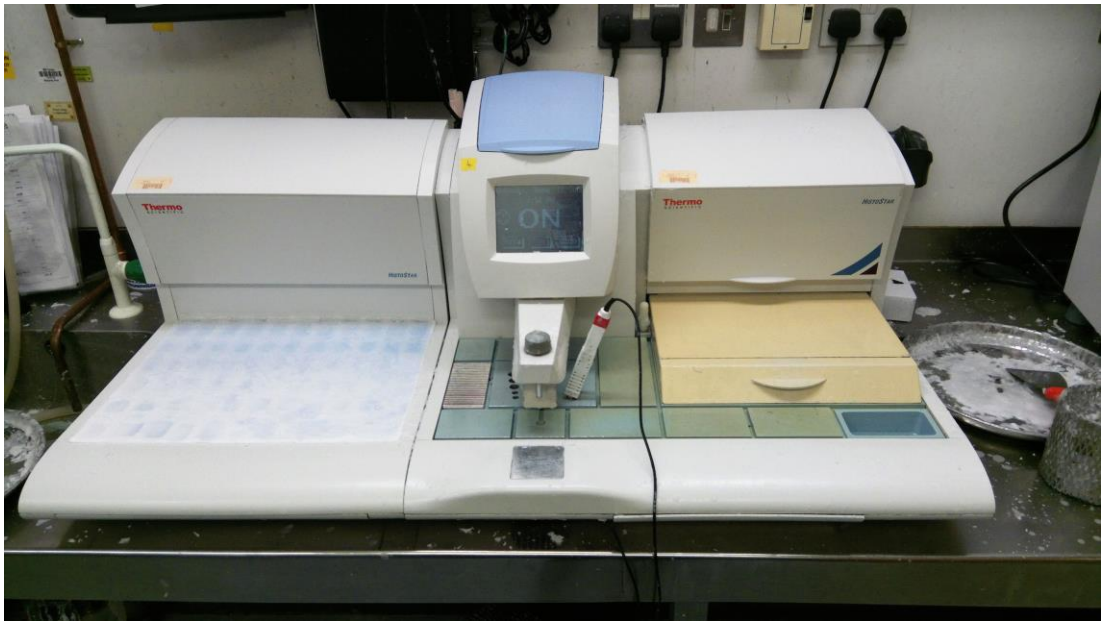


Figure 3.2: Thermo Scientific® HistoStar™ embedding module.

Figure 3.3: LEICA® RM2125 Microtome.

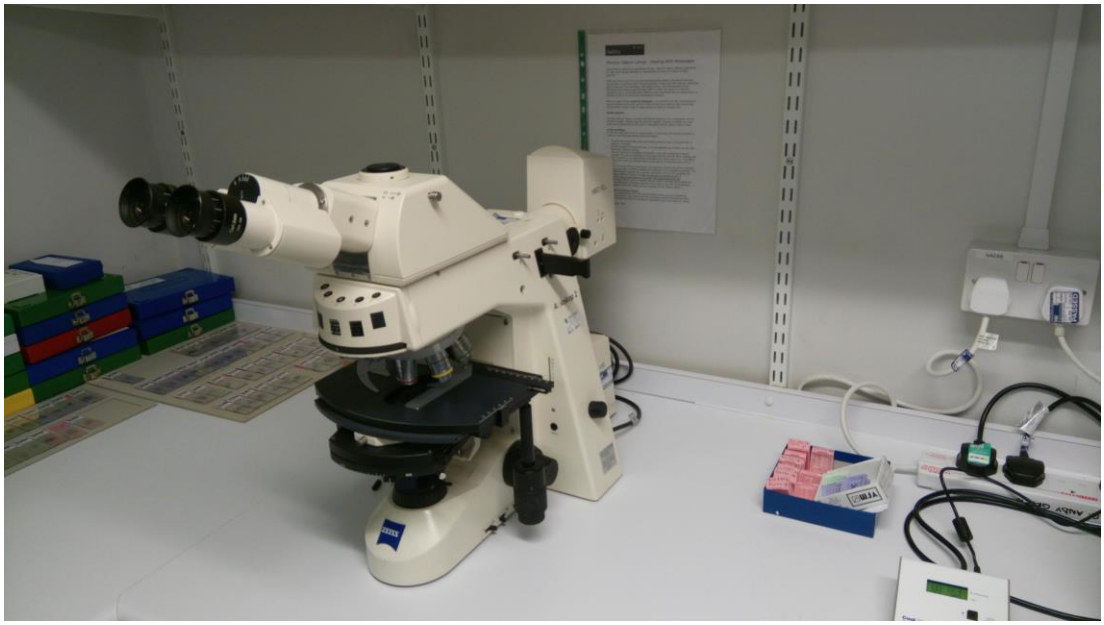


Figure 3.4: Zeiss® Axioskop 2 microscope

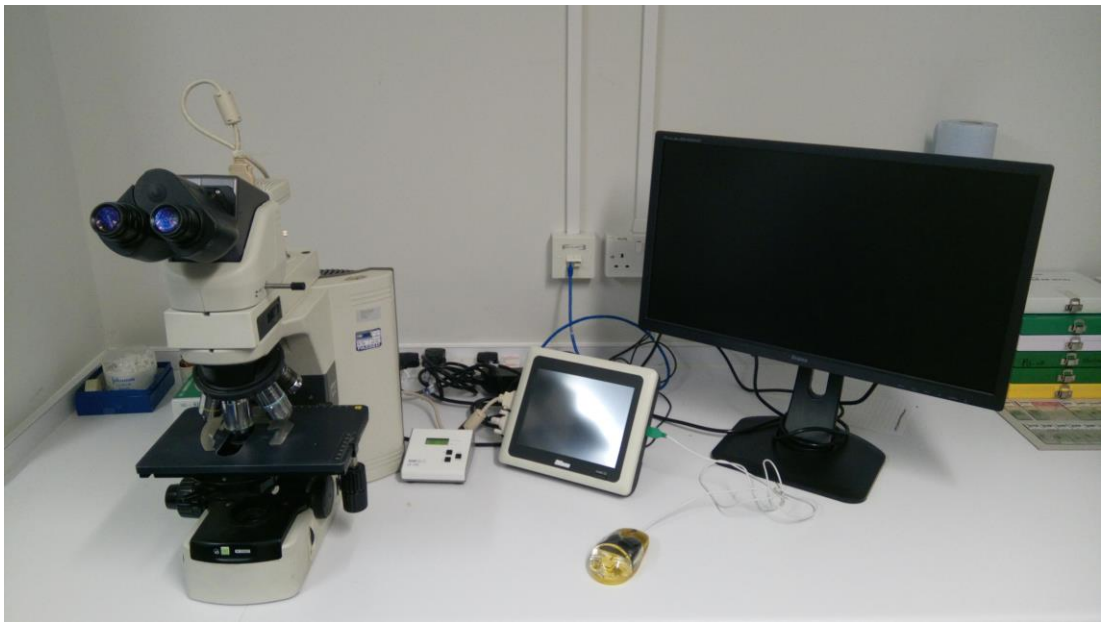


Figure 3.5: Nikon® Eclipse 80i microscope with a Nikon® DS-L3 digital camera system

3.4.2 Primary Antibodies

Four antibodies were used to detect the expression of the chosen inflammatory markers (Table 3.1). They have been validated and guaranteed for IHC of formalin-fixed and paraffin-embedded samples by their commercial suppliers. The optimal

dilution/concentration, incubation time and antigen retrieval of each antibody were optimised for the study's samples. The optimisation process started with the recommended conditions on the antibody datasheets provided by the suppliers. The conditions that were used in previously published studies were also taken into account. The conditions were adjusted accordingly for the optimal staining intensity and clear staining background to be achieved. Positive staining controls were used as recommended by the antibody supplier. Positive control sections were included in every IHC run and stained along with the rest of the slides (Table 3.2). Negative controls were included in each IHC run and demonstrated by omitting the primary antibody.

Beta-2 immunoglobulin was used as a primary antibody to stain one section in each IHC run. However, it was not used to assess the expression of an inflammatory marker as part of the experimental work of this study. Incubation with beta-2 immunoglobulin resulted in an immunostaining of distinctive intensity. The use of beta-2 immunoglobulin allowed the detection of variations in the immunostaining between different IHC runs that would indicate inconsistencies in either the technique or the application of the different substrates of the Novolink™ polymer detection system, Leica® RE7280-K. When the expected intensity, as indicated by the beta-2 immunoglobulin, was not achieved, all the slides of that particular IHC run were discarded and the run was repeated. The use of this method ensured that the immunostaining was consistent.

Table 3.1: Primary antibodies used in this study.

Cellular marker	Primary antibody	Clones	Cellular location	Supplier (Product No)
Serotonin	Mouse monoclonal	5HT-H209	Cytoplasm	Dako (M0758)
TNF-α	Mouse monoclonal	2C8	Cytoplasm	LSBio (LS-B7268)
IL-6	Mouse monoclonal	10C10	Cytoplasm	Leica (NCL-L-IL6)
IL-2R	Mouse monoclonal	4C9	Cell membrane	Leica (NCL-CD25-305)
Beta-2 immunoglobulin	*Mouse polyclonal*	*N/A*	*Cytoplasm*	*Dako (A0072)*

Table 3.2: Details of the optimal immunostaining conditions from optimisation of the primary antibodies used in this study.

Antibodies	Positive control tissue	Antigen retrieval	Dilution/ concentration	Incubation time
Anti-serotonin	Carcinoid	HIER with citrate buffer	1:100	30 minutes at RT
Anti-TNF-α	Inflamed colon	HIER with citrate buffer	7.5 µl/ml	1 hour at RT
Anti-IL-6	Normal colon	HIER with citrate buffer	1:50	1 hour at RT
Anti-IL-2R	Tonsil	HIER with citrate buffer	1:400	Overnight at 4 °C
Anti-β2 immunoglobulin	*N/A*	*HIER with citrate buffer*	*1:2000*	*Same as the main primary antibody*

3.4.3 Immunohistochemical staining procedure

Immunohistochemistry was performed on 5 µm tissue sections. The slides were heated on a 60 °C hotplate (Figure 3.6) for 10 minutes and then loaded to Leica® Autostainer XL (Figure 3.7) for dewaxing and rehydration with xylene and graded alcohol. The slides were subsequently boiled at 98 °C in 0.01 M citrate buffer of pH 6.0 for 20 minutes in a microwave oven [1000-Watt Whirlpool® Jet Stream 359 6th sense (Figure 3.8)] to achieve heat induced epitope retrieval (HIER). After cooling down for 5 minutes under running water, the slides were loaded onto the Shandon Sequenza® coverplates (Figures 3.9 and 3.10). Serial blocking was performed with peroxidase (3-4% hydrogen peroxidase) and protein [0.4% Casein in phosphate-buffered saline (PBS)] for 5 minutes each. The slides were incubated with the primary antibody against the specific cellular protein for the required period time and temperature as determined by optimisation. The Novolink™ polymer detection system, Leica® RE7280-K with HRP-linker antibody conjugates and DAB chromogen was applied for enzyme-substrate labelling. Each substrate was applied for 5 minutes. In a similar way, each section was incubated with DAB for 5 minutes. Following incubation with each of the substrates of the Novolink™ polymer detection system, including DAB, the slides were washed with H_2O twice. Each wash lasted 5 minutes. Following the final wash, haematoxylin was applied for 6 minutes to achieve counterstaing of the sections. A timer was used to ensure accurate time keeping. The Leica® Autostainer XL was used for dehydration of the slides. Finally, the slides were mounted in non-aqueous dibutyl phthalate with xylene (DPX) mounting medium (Appendix-2, IHC procedure).

Figure 3.6: Leica® HI1220 Hotplate.

Figure 3.7: Leica® Autostainer XL.

Figure 3.8: Whirlpool® Jet Stream 359 6th sense microwave oven.

Figure 3.9: Thermo Scientific® Sequenza Coverplates™.

Figure 3.10: Immunostaining using Sequenza Coverplates™ and trays.

3.4.4 Assessment of immunohistochemical staining

Manual IHC assessment was used in this study as some of the inflammatory markers were detected in the membrane and some other in the cytoplasm of the cells and computer-aided scoring would not provide accurate data. This work examined the expression of four inflammatory mediators. The quantitative/semi-quantitative evaluations were done in stained full sections of surgical specimen. The scoring methods that were used to establish the expression/presence of each biological marker in the tissues have been described in previously published studies on the corresponding biological markers. In two of them (TNF-α and IL-2R) the immunostaining was quantified by painstakingly counting the positively stained cells. The quantification of TNF-α was performed simultaneously by the author of this thesis and a senior histopathology consultant, Dr A. M. Zaitoun. The *in-situ* levels of serotonin were also simultaneously semi-quantified by the author of this thesis and Dr A. M. Zaitoun using the H-scoring system. Both researchers had to agree on the score given to each slide. The expression of IL-6 was semi-quantified by the author of this thesis using the H-scoring system. 25% of the slides stained for IL-6 and IL-2R were independently assessed by Dr A. M. Zaitoun. As the differences in the scores between the two researchers were not significant, the scores obtained by the author of this thesis were used in the final analysis. Moreover, during the scoring process, both the clinical and histopathological characteristics of each slide were masked from the observers in order to avoid introduction of bias.

3.4.4.1 Semi quantification of serotonin positive cells

Ten non-overlapping fields were selected and examined at ×200 magnification by two observers simultaneously. Positive staining was observed in the cytoplasm of the enterochromaffin and subepithelial neuroendocrine cells of the mucosa. The amount of serotonin in the specimen was semi-quantified using a modified H-scoring system. The total number of immunostained cells of each field was counted and multiplied by a number representing the intensity (1 for weak, 2 for moderate and 3 for strong). Both observers had to agree on the score that was given to each field. The average of the scored fields for each cell population was calculated and formed the immunohistochemical score of the specimen for the particular cell population. Samples with less than three of ×200 magnification fields were excluded from the analysis.

3.4.4.2 Quantification of TNF-α positive cells

Ten non-overlapping fields were selected in the mucosa of each section and examined at high-power magnification (×400) by two observers simultaneously. Positive staining for TNF-α was observed as dark-brown cytoplasm of mononuclear cells. The positively stained cells of each high-power field were counted, and the average of the scored fields was calculated and formed the immunohistochemical score of the specimen. Specimens with less than four high-power fields were excluded from the analysis [268, 269].

3.4.4.3 Semi-quantification of IL-6 positive cells

Ten non-overlapping fields were selected in the mucosa and examined at high-power magnification (×400). Positive staining was observed in the cytoplasm of epithelial and inflammatory cells. The expression of IL-6 was semi-quantified using the H-scoring system. The average of the scored fields was calculated and formed the immunohistochemical score of the specimen. Samples with less than four high-power fields were excluded from the analysis. 25% of the slides were independently assessed by Dr A. M. Zaitoun. As the differences in the scores between the two researchers were not significant, the scores obtained by the author of this thesis were used in the analysis.

3.4.4.4 Quantification of IL-2R positive cells

Ten non-overlapping fields were selected in the mucosa and submucosa and examined at high-power magnification (×400). Positive staining was observed in the membrane of lymphocytes. The positively stained cells of each high-power field were counted, and the average number of positively stained cells was calculated for the mucosa and submucosa of each section. In the submucosa, the germinal centres were avoided due to the extremely high number of positive cells. Specimens with less than three high-power fields were excluded from the analysis. 25% of the slides were independently assessed by Dr A. M. Zaitoun. As the differences in the scores between the two researchers were not significant, the scores obtained by the author of this thesis were used in the analysis.

3.4.5 Statistical analysis

Statistical analysis was performed using the IBM SPSS statistics software, version 22 (SPSS Inc, Chicago, IL, USA). Where data did not follow a normal distribution (Shapiro-Wilk test, $p<0.05$), non-parametric tests [Mann-Whitney U test (between two groups) and Kruskal-Wallis H test (between three or more groups)] were used to compare the differences between the groups based on histopathological parameters. Cross tabulation and the Pearson's chi-square test were used for categorical variables. The results were considered as statistically significant when the probability value (p value) was less than 0.05 ($p<0.05$, 2 tailed). In order to avoid Type I error, Bonferroni correction was applied when multiple comparisons were performed. Bonferroni correction is calculated by dividing the p value by the number of comparisons. Tukey's boxplots (box and whisker plots) were created on GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA).

3.5 Challenges and technical difficulties

During the project several challenges had to be overcome. Difficulties were encountered in all stages, from acquiring significant number of slides while ensuring that inclusion and exclusion criteria were met, to technical difficulties with the immunostaining, as well as funding issues.

The first challenge that the research team had to overcome was the amount of time that was needed to acquire the required number of slides from the archive of the Department of Histopathology at the Queen's Medical Centre. According to the design of the study, each group should include at least 100 patients and given the size of the centre the study was conducted in, this task seemed to be a reasonably easy to accomplish. No issues were encountered in acquiring the required number of slides for the acute uncomplicated appendicitis group. However, several difficulties were encountered during the collection of the slides for the other groups. Although, every effort was made to include consecutive samples, some of the specimens were already taken for other projects. This issue was especially apparent in the collection of appendix specimens for the control group. Many of the slides containing normal appendices, which were resected as part of elective colectomy, were missing from the archive as they were used by other researchers for projects dealing with bowel cancer. Regarding the group of histologically "normal" appendices, it was challenging to acquire the desired number of 100 samples as some of the samples retrieved from the archive had to be excluded from the study. Although the histopathology report on the electronic database stated that the appendix was normal due to absence of feature of inflammation, a careful review of several sections by a second

histopathologist revealed some evidence of inflammation in some areas of the appendix. Therefore, these slides were excluded as according to inclusion criteria the initial diagnosis that was stated on the histopathology report had to match with the second review by Dr A. M. Zaitoun who is a senior histopathology consultant. It was also very challenging to identify slides with evidence of necrosis or perforation that would be suitable for the study. Slides with extensive necrosis had to be excluded as they have not had enough areas to score the immunostaining. Moreover, significant amount of calcification would affect the thickness of the slides as well as the immunostaining and the samples had to be excluded. As a consequence, a big number of slides that were initially retrieved from the archive were excluded which resulted in further electronic searches in the database and several visits to the archive to collect more samples. Thus, the collection of the required number of slides according to inclusion and exclusion criteria, while maintaining the quality standards of the project as high as possible, required significant amount of time that was not anticipated at the initial stages of the study.

Another aspect of the study that proved to be very challenging was the retrieval of a big number of patient notes from the case note library of the hospital. The patient notes had to be requested in small batches according the prescribed protocol of Nottingham University Hospitals NHS Trust. The research team encountered significant delays in retrieving some of the notes, as the use of the case notes for clinical reasons took priority over the use for research purposes. Moreover, the extraction of significant amount of data from a large number of patient notes was

very time consuming. Therefore, substantial amount of time was needed to collect the required clinical data for the current study.

During this project considerable challenges in several aspects of the laboratory work had to be overcome. First and foremost, several hours of training by certified technicians of the immunohistochemistry laboratory of the University of Nottingham were needed, in order for the author of this thesis to be able to successfully perform all the technical aspects in preparation of the experimental work such as melting and re-embedding tissue blocks as well as sectioning tissue blocks. Moreover, several training immunohistochemistry runs were performed under the close supervision of experienced technicians in order to achieve consistency. The results were assessed by Dr A. M. Zaitoun and only when the required experience was achieved the experimental work was performed by the author of this thesis.

Another difficulty that was encountered during the laboratory work was the consistency in the intensity of the immunostaining between different immunohistochemistry runs. To overcome this challenge, immunostaining with β2 immunoglobulin was used as described in the chapter 3.4.2 Primary antibodies. When the intensity of the β2 immunoglobulin immunostaining between different immunohistochemistry runs for the same inflammatory marker was not the same, the slides were discarded, and the immunostaining was performed again. Once the slides were re-stained, the intensity was examined by Dr A. M. Zaitoun to ensure that consistency was achieved. Following a satisfactory review by Dr A. M. Zaitoun the slides were scored.

Moreover, as it will become apparent later in this thesis, some of the results, such as the significantly low expression of TNF-α and IL-6 of the acute uncomplicated appendicitis samples, were not anticipated. In order to validate the observations and assess the reproducibility of the results, the immunohistochemistry runs with each specific antibody were repeated and the slides scored again by two researchers. The differences between the initial and the repeat immunohistochemistry runs were not significantly different and therefore, the scores of the initial immunohistochemistry runs were kept for the analysis.

Finally, financial difficulties were encountered during this project as the funding available to the research team was very limited due to unforeseen circumstances. With the limited budget available, some of the technical aspects of the experimental work such as the re-embedding and sectioning of blocks could not be performed by University of Nottingham or NHS technicians as the quoted cost was not possible to be covered. Should the research team was able to afford this, the experimental work would have been completed a lot sooner. Moreover, the limited funding prevented the research team from exploring the use of other techniques such as gene analysis and qPCR to measure mRNA expression. Although these techniques, would provide additional data to help the understanding of the pathogenesis as well as the immune response in the different aspects of appendicitis, are very costly and in the present study, given the number of patients included, the cost would rise to levels well above the available budget.

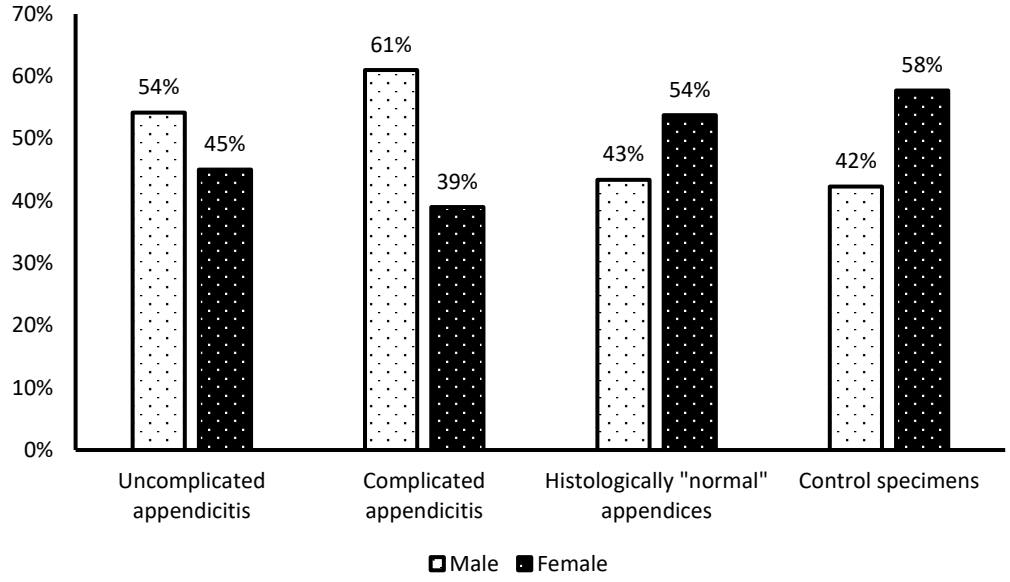
CHAPTER 4: Results

4.1 Demographics

As it has been described in CHAPTER 3: Materials and methods, the samples that were collected from the archive of the Department of Histopathology at Nottingham University Hospitals NHS Trust, were allocated into four different groups following conventional histopathological examination. Group I included 120 appendix specimens exhibiting features of uncomplicated acute appendicitis (median age 30.0, IQ range 22.0-45.0). Group II included 118 appendix specimens with features of complicated acute appendicitis (median age 34.0, IQ range 21.0-49.2), whereas group III included 104 appendix specimens with no features of inflammation on conventional microscopy (histologically "normal") specimens (median age 22.0, IQ range 18.0-29.0). The control group (Group IV) was consisted of appendices removed as part of the resected colon (n=106) for pathology other than appendicitis as described in the inclusion and exclusion criteria section (median age 70.5, IQ range 61.0-79.0). The uncomplicated acute appendicitis group included 65 (54%) male and 54 (45%) female patients. The complicated acute appendicitis group included 72 (61%) male and 46 (39%) female patients. The group of histologically "normal" specimens included 44 (43%) male and 60 (54%) female patients. The control group included 46 (42%) male and 57 (58%) female patients (Table 4.1, graph 4.1).

Table 4.1: Summary of the population and the demographics of the patients between the 4 groups of the study. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients in each group. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range.

		Gender		Age			
Groups	**N**	**Male**	**Female**	**Median**	**25th**	**75th**	**IQ range**
I	120	65	54	30.0	22.0	45.0	23.0
II	118	72	46	34.0	21.0	49.2	28.2
III	104	44	60	22.0	18.0	29.0	11.0
IV	106	46	57	70.5	61.0	79.0	18.0



Graph 4.1: Summary of data on the gender of patients between the 4 groups of the study. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV patients of the control group.

4.2 Experimental data

4.2.1 Serotonin content

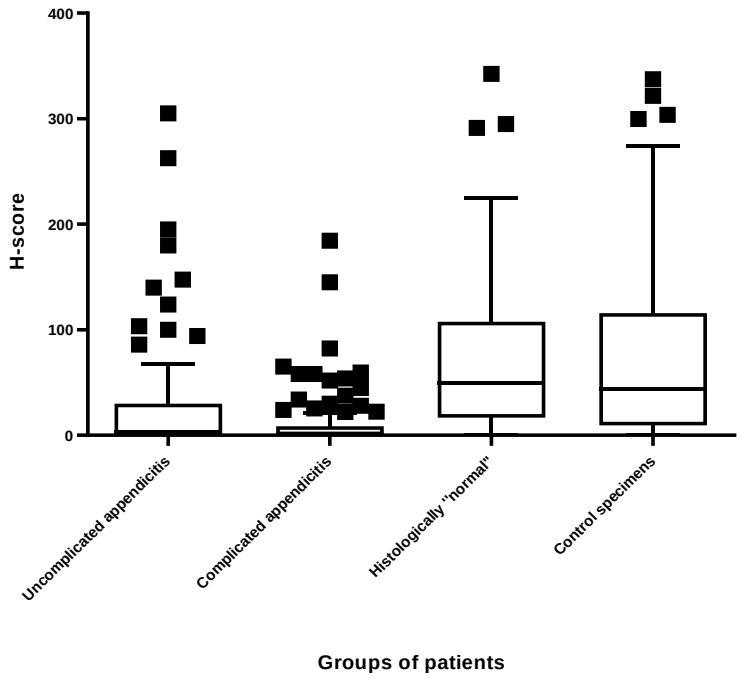
Immunohistochemistry performed with anti-serotonin antibody demonstrated enterochromaffin cells within the crypts of the epithelium. Immunostaining also revealed subepithelial neuroendocrine cells in the *lamina propria*. Subepithelial neuroendocrine cells appeared either as solitary cells or small clusters close to the crypts.

4.2.1.1 Serotonin contents of enterochromaffin cells

Serotonin contents of enterochromaffin cells ware assessed on a total of 437 specimens as 11 samples did not have at least 3 fields with ×200 magnification and therefore excluded from the study. Enterochromaffin cells of both uncomplicated acute (median 3.0, IQ range 0-30.0, $p<0.001$) and complicated acute (median 0, IQ range 0-8.5, $p<0.001$) appendicitis specimens were markedly depleted of serotonin compared with the control group (median 43.5, IQ range 9.5-115.8). The amount of serotonin contained by enterochromaffin cells of histologically "normal" appendices (median 49.7, IQ range 16.7-107.5) did not differ significantly compared with the control group (median 43.5, IQ range 9.5-115.8, $p=0.60$). Both group I (median 3.0, IQ range 0-30.0, $p<0.001$) and II (median 0, IQ range 0-8.5, $p<0.001$) contained significantly less serotonin compared with group III (median 49.7, IQ range 16.7-107.5). Moreover, enterochromaffin cells of complicated acute appendicitis (median 0, IQ range 0-8.5) samples were significantly depleted compared with acute uncomplicated appendicitis (median 3.0, IQ range 0-30.0, $p=0.001$) samples (Table 4.2, graph 4.2, figures 4.1-4.12).

Table 4.2: Summary of the analysis of serotonin contents of enterochromaffin cells between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens; Group II-complicated appendicitis specimens; Group III-histologically "normal" appendices; Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Group	N	Median	25th	75th	IQ range	p value
I	115	3.0	0.0	30.0	30.0	<0.001
II	113	0.0	0.0	8.5	8.5	
III	104	49.7	16.7	107.5	90.8	
IV	105	43.5	9.5	115.8	106.3	



Graph 4.2: Tukey's boxplots displaying data of serotonin contents of enterochromaffin cells between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 3.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 30.0, interquartile (IQ) range 30.0; Group II-complicated appendicitis specimens, median 0.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 8.5, interquartile (IQ) range 8.5; Group III-histologically "normal" specimens, median 49.7, 25th percentile (Q1) 16.7, 75th percentile (Q3) 107.5, interquartile (IQ) range 90.8; Group IV-control specimens, median 43.5, 25th percentile (Q1) 9.5, 75th percentile (Q3) 115.8, interquartile (IQ) range 90.8. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1-1.5 × IQ range. Values plotted with ▪ represent outliers.

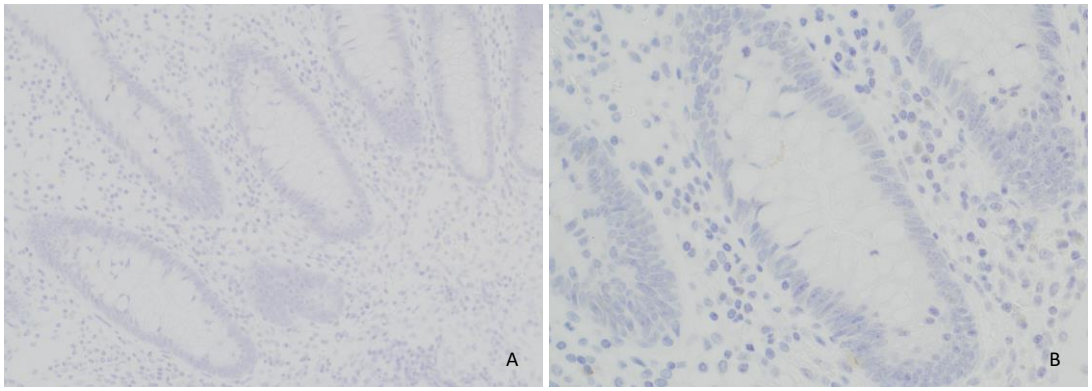


Figure 4.1: Serotonin contents (very low/negative) of enterochromaffin cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

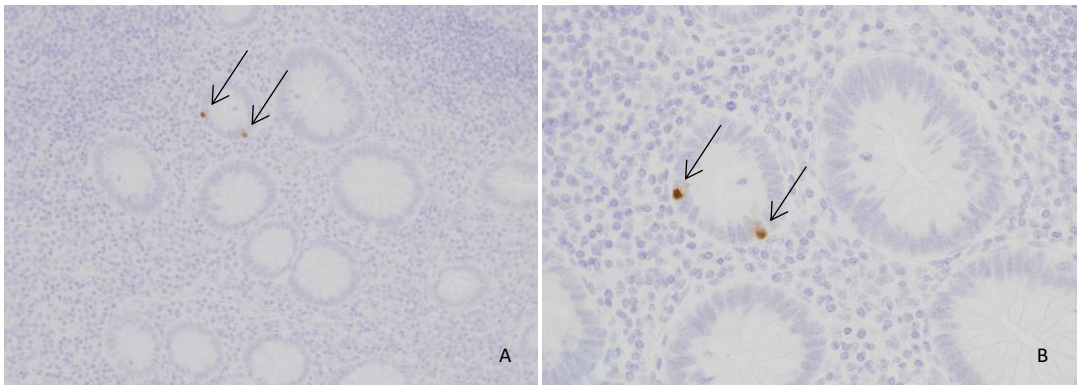


Figure 4.2: Serotonin contents (moderate) of enterochromaffin cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 5.

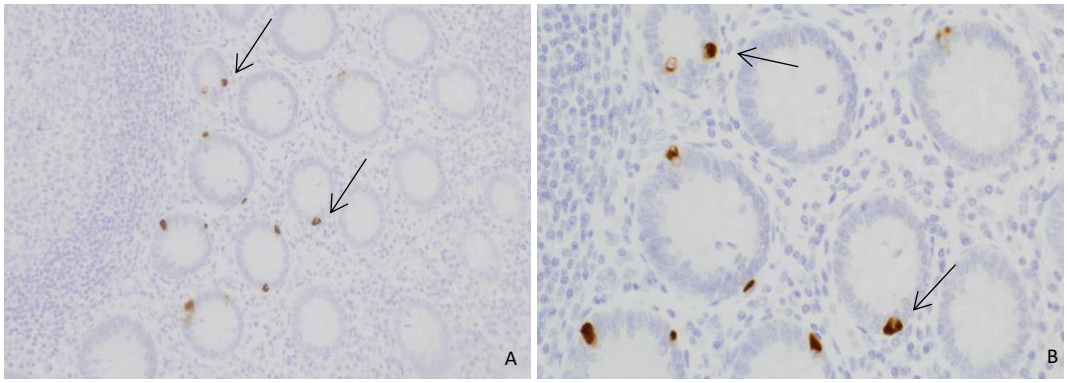


Figure 4.3: Serotonin contents (very high) of enterochromaffin cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 262.5.

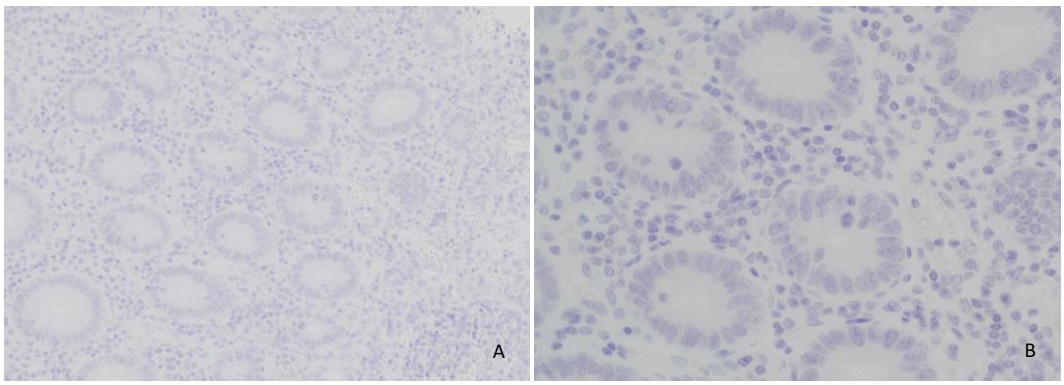


Figure 4.4: Serotonin contents (negative) of enterochromaffin cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

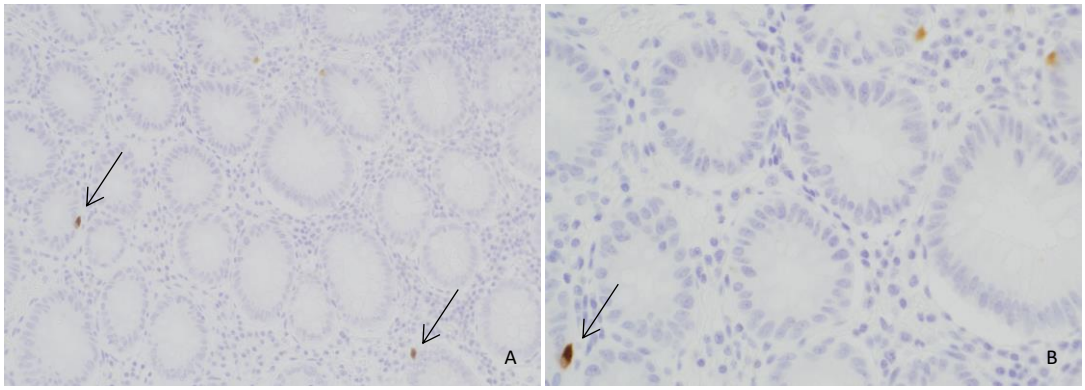


Figure 4.5: Serotonin contents (moderate to high) of enterochromaffin cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 4.

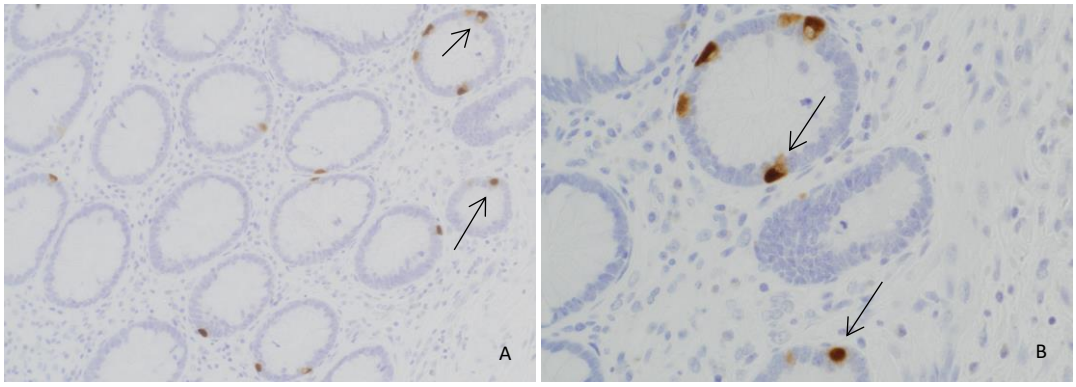


Figure 4.6: Serotonin contents (very high) of enterochromaffin cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 58.

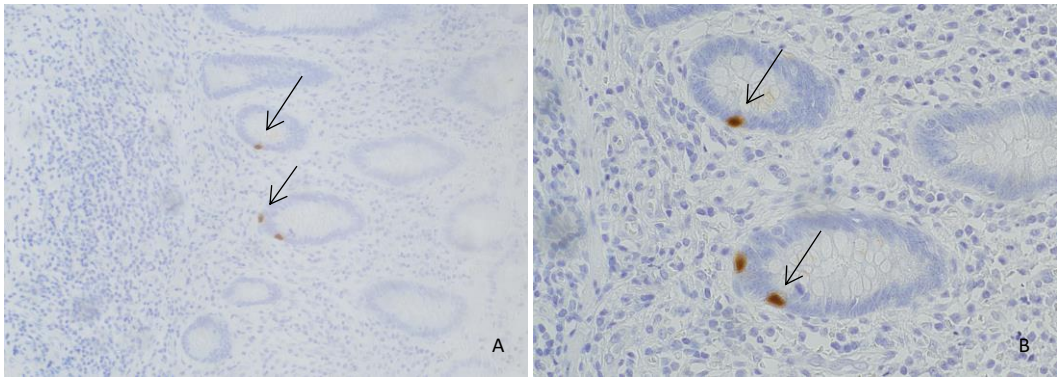


Figure 4.7: Serotonin contents (low) of enterochromaffin cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 16.5.

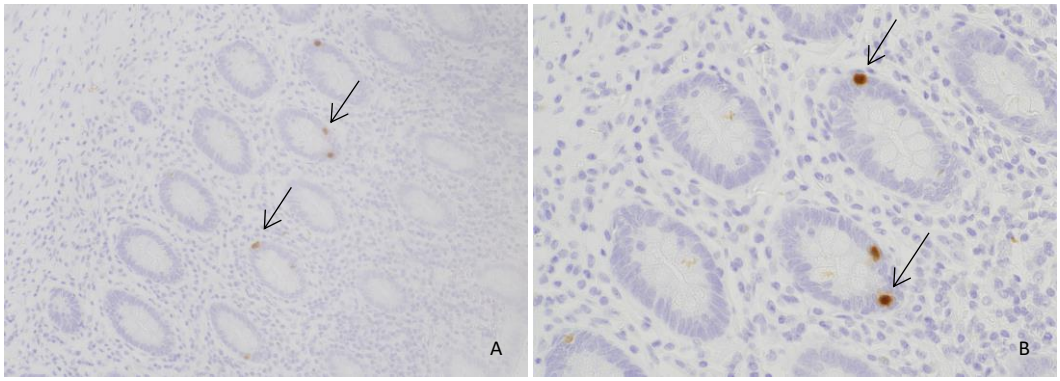


Figure 4.8: Serotonin contents (moderate) of enterochromaffin cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 55.9.

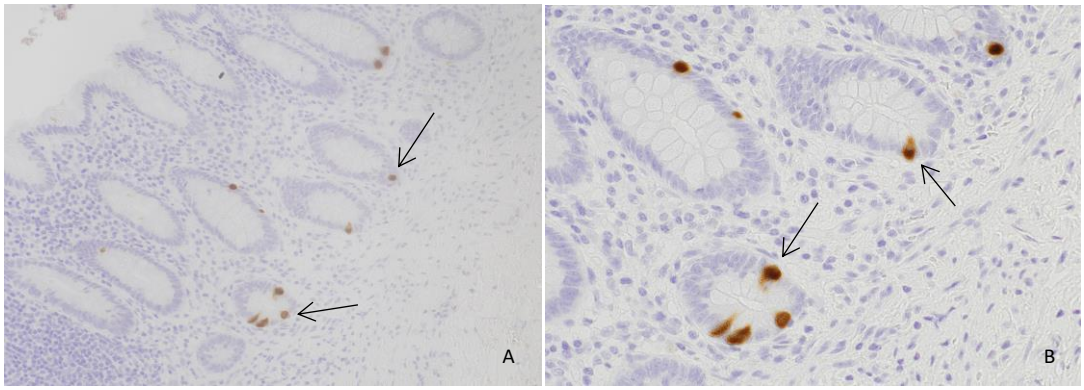


Figure 4.9: Serotonin contents (high) of enterochromaffin cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 342.5.

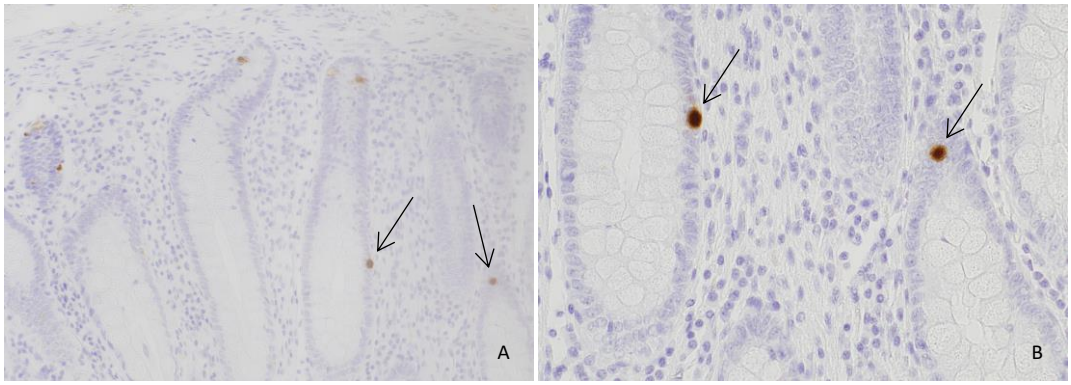


Figure 4.10: Serotonin contents (low) of enterochromaffin cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 18.5.

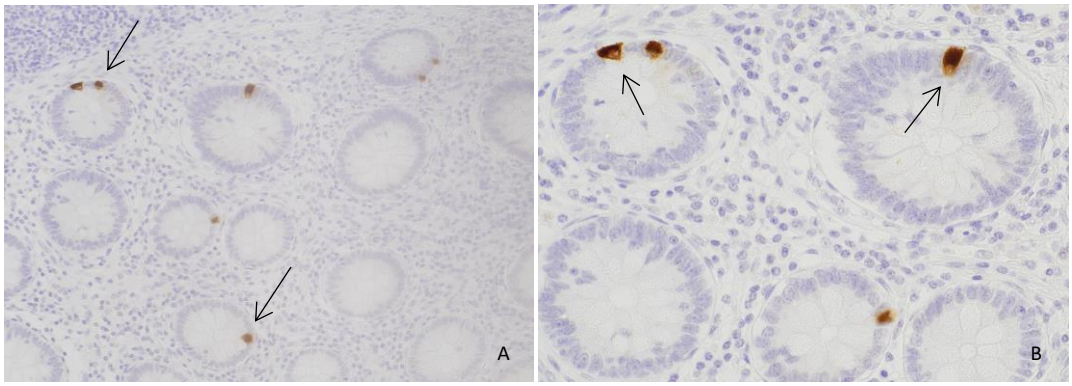


Figure 4.11: Serotonin contents (moderate) of enterochromaffin cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 124.

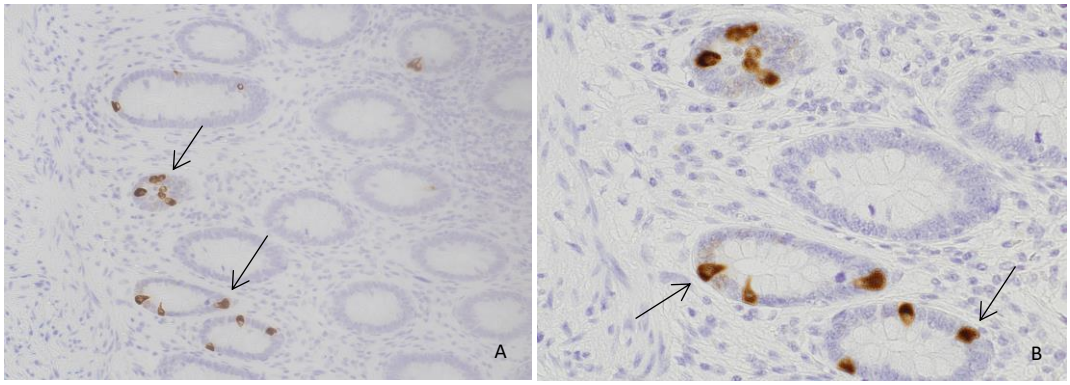


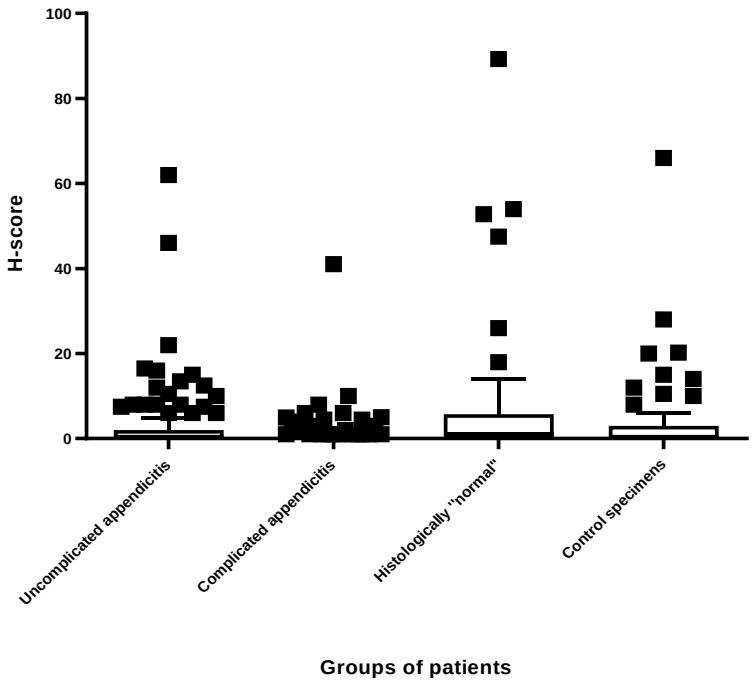
Figure 4.12: Serotonin contents (high) of enterochromaffin cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of enterochromaffin cells. The positive brown cytoplasmic staining indicates enterochromaffin cells within the crypts of the epithelium (arrows). The H-score for this sample was 337.5.

4.2.1.2 Serotonin contents of subepithelial neuroendocrine cells

Serotonin contents of subepithelial neuroendocrine cells were assessed on a total of 446 specimens as 2 samples did not have at least 3 fields with ×200 magnification and therefore excluded from the study. Subepithelial neuroendocrine cells of complicated appendicitis (median 0, IQ range 0-0) samples were significantly depleted of serotonin compared with the control group (median 0, IQ range 0-3.0, $p<0.001$). The amount of serotonin contained by subepithelial neuroendocrine cells of acute uncomplicated appendicitis (median 0, IQ range 0-2.0, $p=0.39$) samples and histologically "normal" appendices (median 1.0, IQ range 0-5.7, $p=0.15$) did not differ significantly compared with the control group (median 0, IQ range 0-3.0). Subepithelial neuroendocrine cells of complicated acute appendicitis (median 0, IQ range 0-0) contained significantly less serotonin compared with acute uncomplicated appendicitis (median 0, IQ range 0-2.0, $p=0.004$) samples and histologically "normal" appendices (median 1.0, IQ range 0-5.7, $p<0.001$). However, subepithelial neuroendocrine cells of acute uncomplicated appendicitis (median 0, IQ range 0-2.0) samples contained significantly more serotonin compared with the histologically "normal" appendices (median 1.0, IQ range 0-5.7, $p=0.001$) (Table 4.3, graph 4.3, figures 4.13-4.24).

Table 4.3: Summary of the analysis of serotonin contents of subepithelial neuroendocrine cells between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	120	0.0	0.0	2.0	2.0	<0.001
II	117	0.0	0.0	0.0	0.0	
III	104	1.0	0.0	5.7	5.7	
IV	105	0.0	0.0	3.0	3.0	



Graph 4.3: Tukey's boxplots displaying data of serotonin contents of subepithelial neuroendocrine cells between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 0.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 2.0, interquartile (IQ) range 2.0; Group II-complicated appendicitis specimens, median 0.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 0.0, interquartile (IQ) range 0.0; Group III-histologically "normal" specimens, median 1.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 5.7, interquartile (IQ) range 5.7; Group IV-control specimens, median 0.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 3.0, interquartile (IQ) range 3.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1-1.5 × IQ range. Values plotted with ▪ represent outliers.

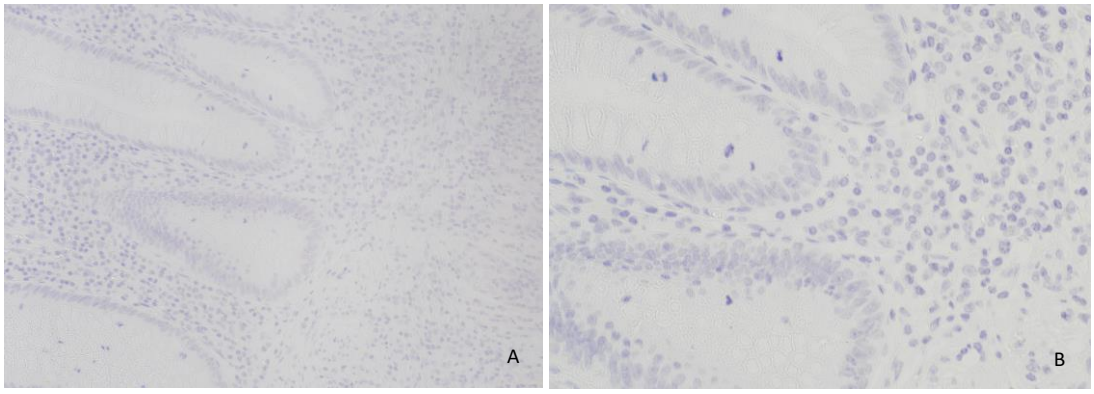


Figure 4.13: Serotonin contents (very low/negative) of subepithelial neuroendocrine cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

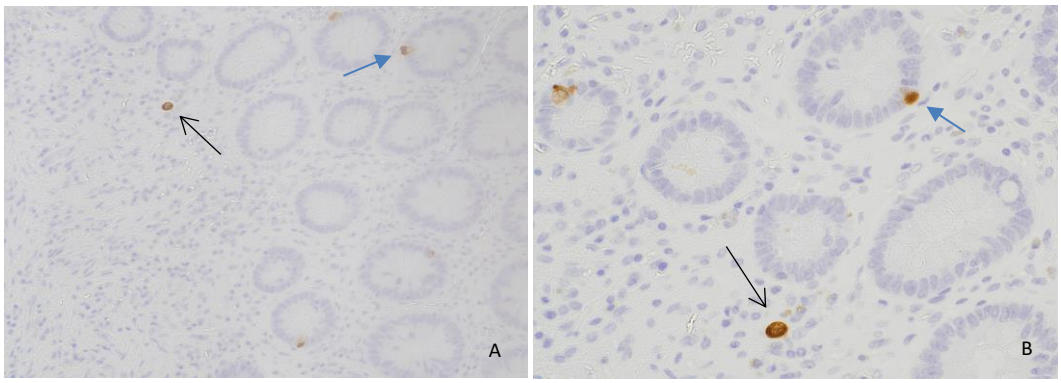


Figure 4.14: Serotonin contents (moderate) of subepithelial neuroendocrine cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 1.

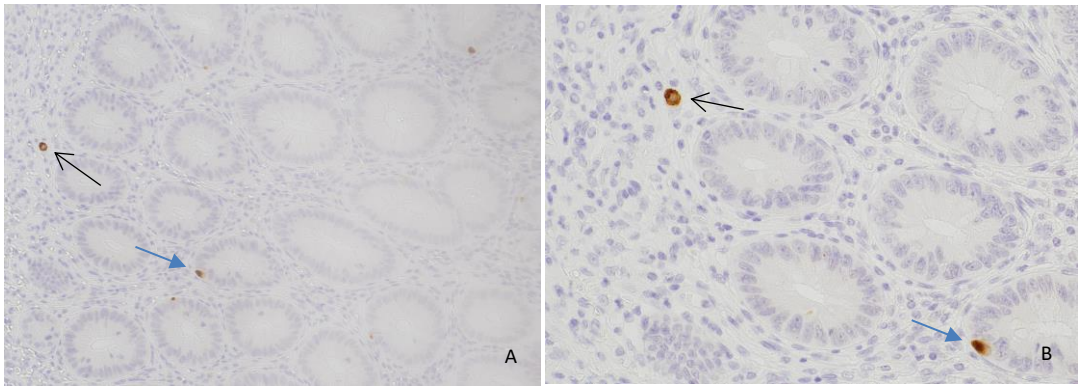


Figure 4.15: Serotonin contents (high) of subepithelial neuroendocrine cells in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 12.

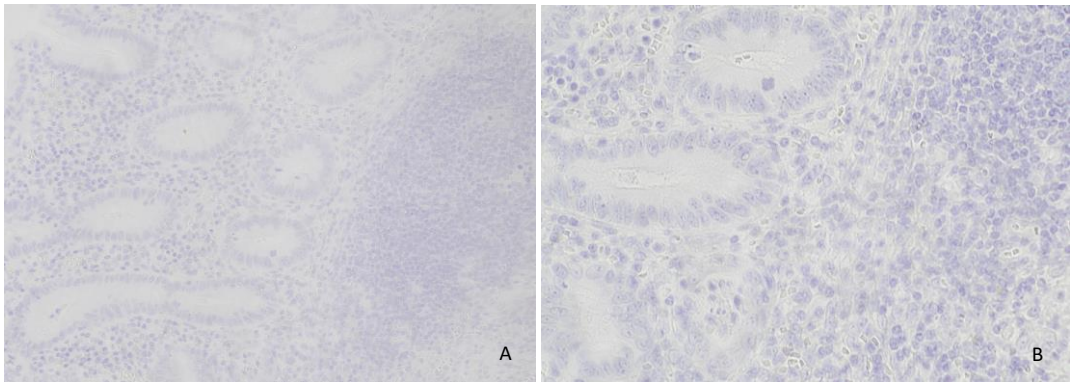


Figure 4.16: Serotonin contents (very low/negative) of subepithelial neuroendocrine cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

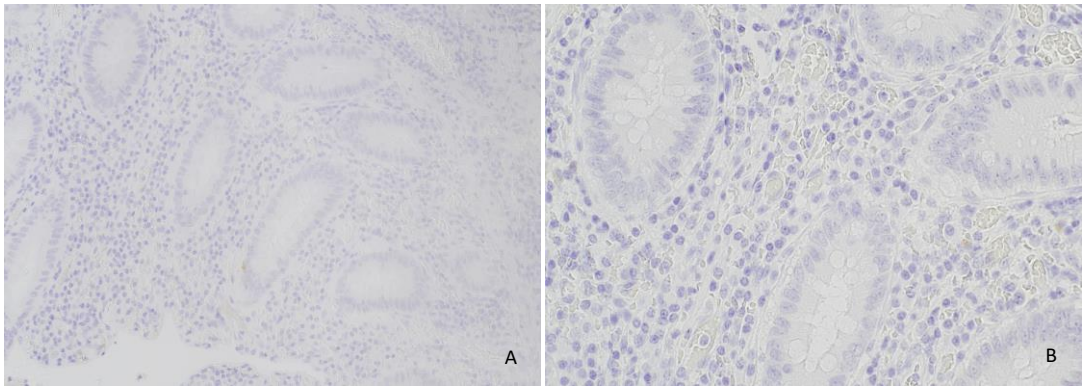


Figure 4.17: Serotonin contents (low/moderate) of subepithelial neuroendocrine cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

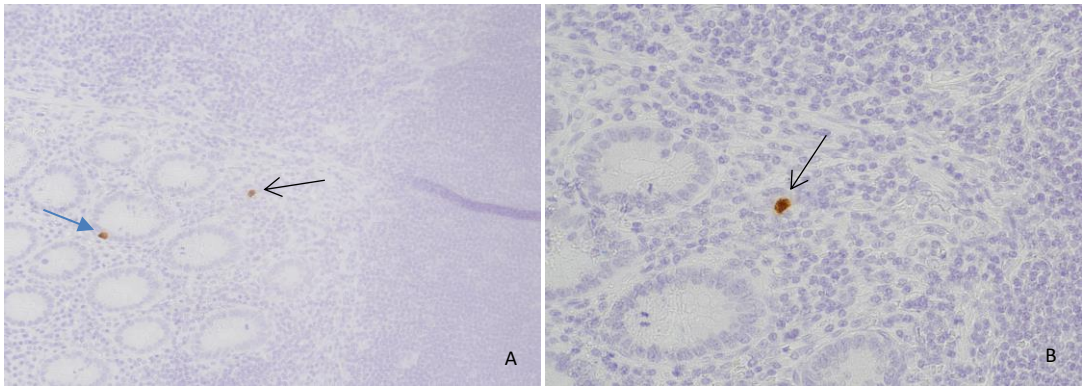


Figure 4.18: Serotonin contents (high) of subepithelial neuroendocrine cells in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 4.5.

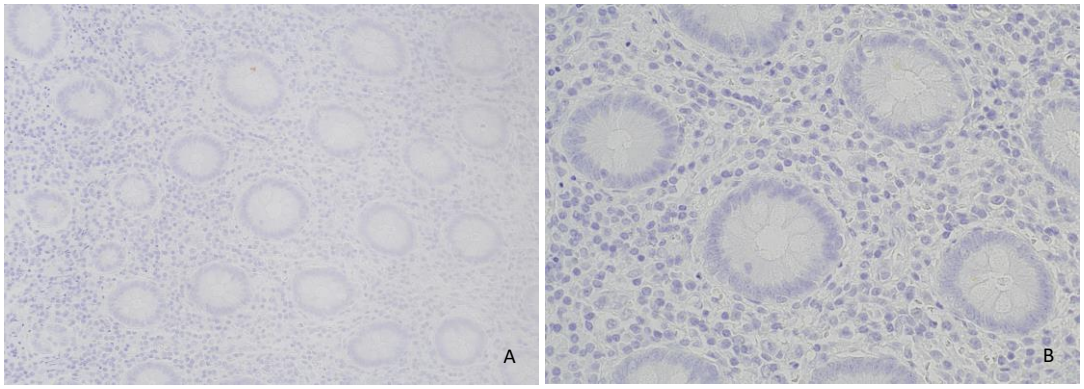


Figure 4.19: Serotonin contents (very low/negative) of subepithelial neuroendocrine cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

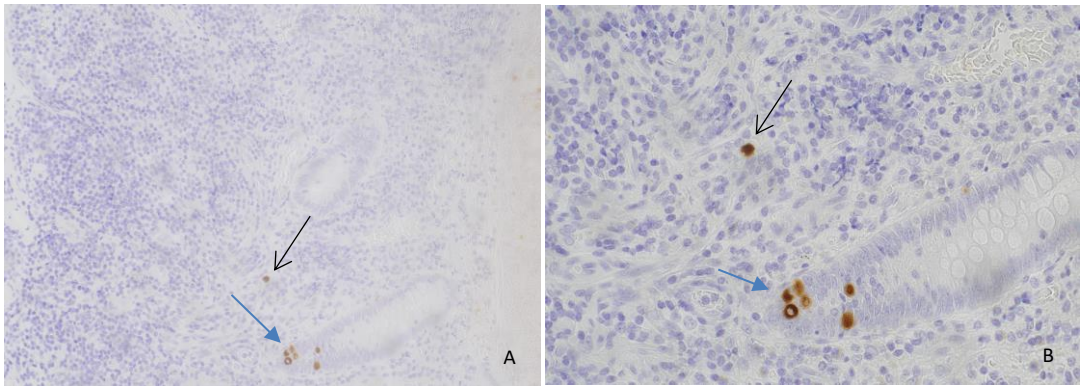


Figure 4.20: Serotonin contents (moderate to high) of subepithelial neuroendocrine cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 3.

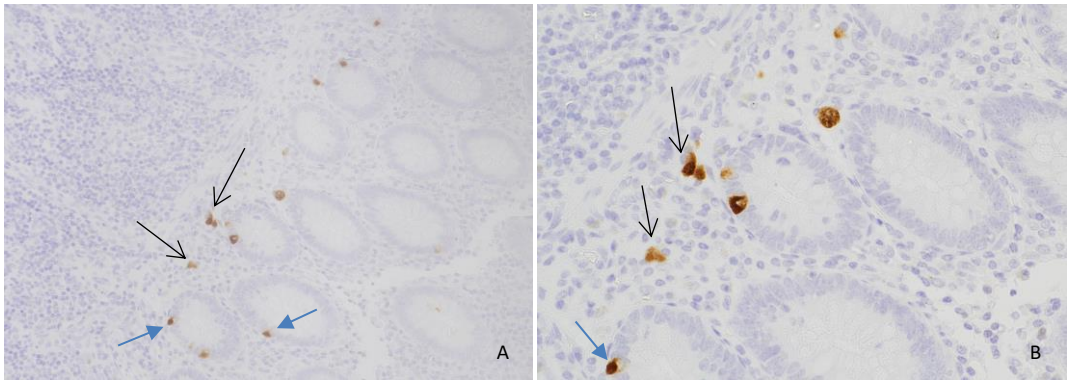


Figure 4.21: Serotonin contents (high) of subepithelial neuroendocrine cells in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 14.

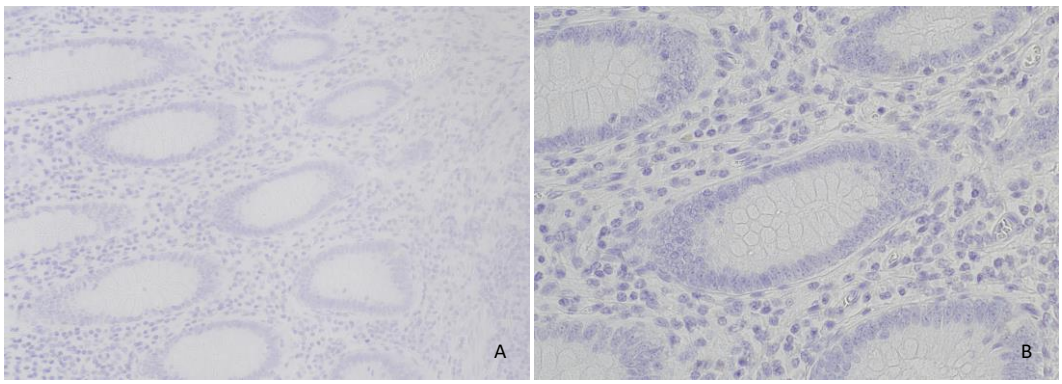


Figure 4.22: Serotonin contents (very low/negative) of subepithelial neuroendocrine cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The absence of positive brown cytoplasmic-stained cells indicates that the sample was severely depleted of serotonin. The H-score for this sample was 0.

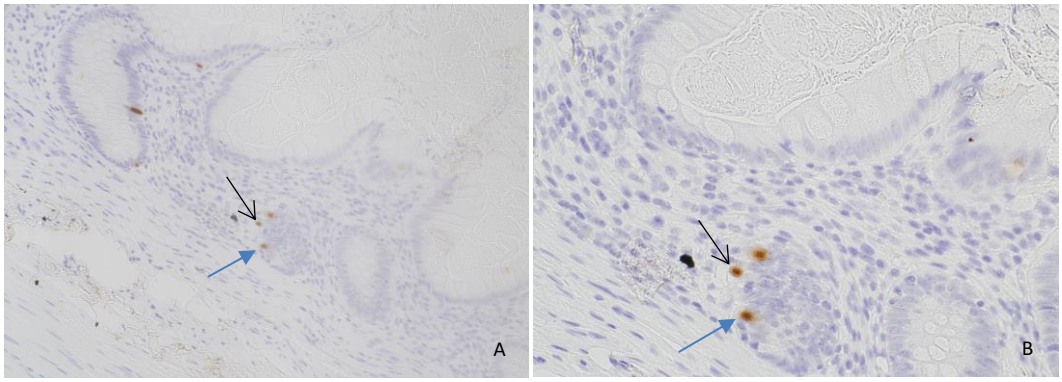


Figure 4.23: Serotonin contents (moderate) of subepithelial neuroendocrine cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 2.

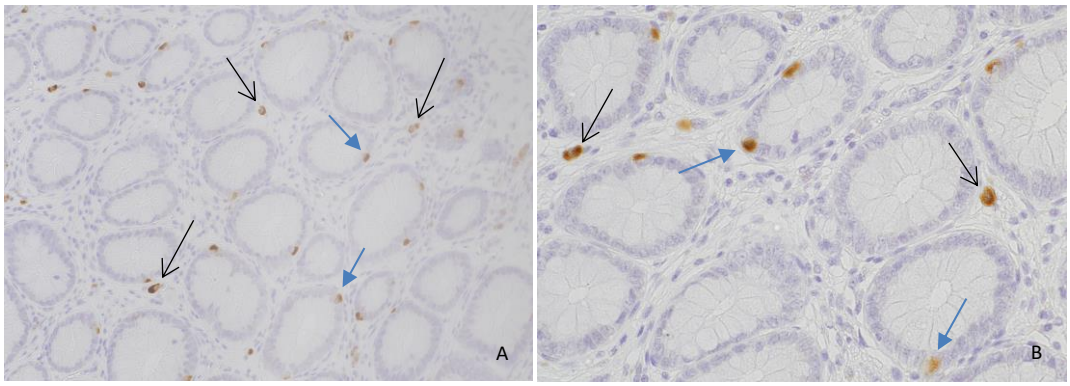


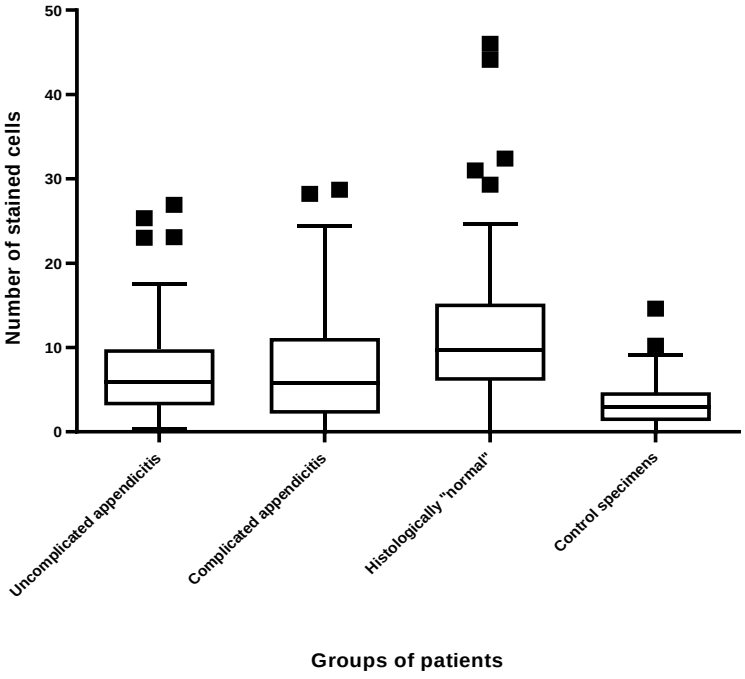
Figure 4.24: Serotonin contents (high) of subepithelial neuroendocrine cells in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to serotonin (Dako, M0758) at a 1:100 dilution for 30 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The modified H-score [number of positive cells (brown cytoplasmic-stained cells) × intensity of staining (1 to 3)] was used to assess the serotonin contents of subepithelial neuroendocrine cells. The positive brown cytoplasmic staining indicates subepithelial neuroendocrine cells (black arrows) and enterochromaffin cells (blue arrows). The H-score for this sample was 20.25.

4.2.2 Expression of TNF-α

Immunohistochemistry performed with anti-TNF-α antibody demonstrated monocytes in the mucosa of the samples. TNF-α expression was assessed on a total of 417 specimens as 31 samples did not have at least four high-power fields and therefore excluded from the study. Both groups of acute uncomplicated (median 5.9, IQ range 3.1-9.8, $p<0.001$) and complicated (median 6.8, IQ range 3.6-12.1, $p<0.001$) appendicitis demonstrated significantly increased expression of TNF-α compared with the control group (median 3.0, IQ range 1.4-4.7). The expression of TNF-α in the histologically "normal" appendices (median 9.8, IQ range 6.2-15.2) was also significantly increased compared with the control group (median 3.0, IQ range 1.4-4.7, $p<0.001$). Moreover, the TNF-α expression of the acute uncomplicated appendicitis (median 5.9, IQ range 3.1-9.8) samples was significantly reduced compared with the histologically "normal" (median 9.8, IQ range 6.2-15.2, $p<0.001$) samples as was the complicated appendicitis (median 6.8, IQ range 3.6-12.1) compared with the histologically "normal" appendices (median 9.8, IQ range 6.2-15.2, $p=0.004$). However, TNF-α expression between group I (median 5.9, IQ range 3.1-9.8) and group II (median 6.8, IQ range 3.6-12.1, $p=0.17$) did not vary significantly (Table 4.4, graph 4.4, figures 4.25-4.36).

Table 4.4: Summary of the analysis of TNF-α expression between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	109	5.9	3.2	9.9	6.6	<0.001
II	102	6.8	3.6	12.1	8.4	
III	102	9.8	6.2	15.2	9.0	
IV	104	3.0	1.4	4.7	3.3	



Graph 4.4: Tukey's boxplots displaying data of TNF-α expression between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 5.9, 25th percentile (Q1) 3.2, 75th percentile (Q3) 9.9, interquartile (IQ) range 6.6; Group II-complicated appendicitis specimens, median 6.8, 25th percentile (Q1) 3.6, 75th percentile (Q3) 12.1, interquartile (IQ) range 8.4; Group III-histologically "normal" specimens, median 9.8, 25th percentile (Q1) 6.2, 75th percentile (Q3) 15.2, interquartile (IQ) range 9.0; Group IV-control specimens, median 3.0, 25th percentile (Q1) 1.4, 75th percentile (Q3) 4.7, interquartile (IQ) range 3.3. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1-1.5 × IQ range. Values plotted with ▪ represent outliers.

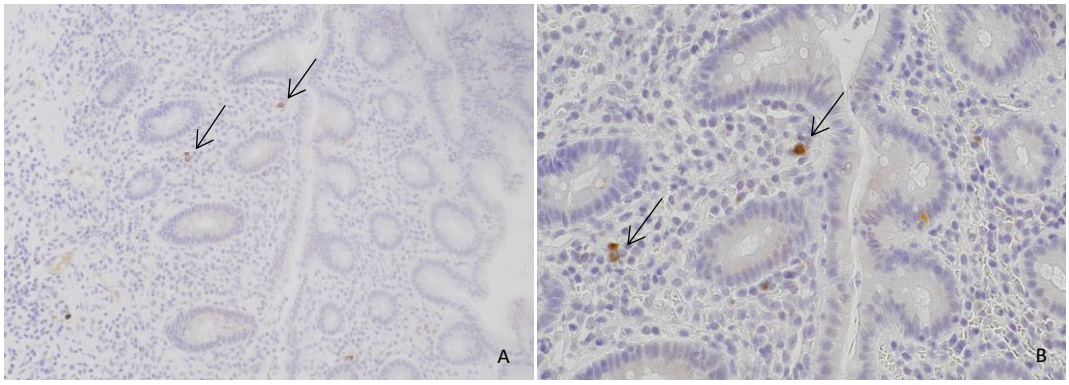


Figure 4.25: Expression of TNF-α (low) in monocytes of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 3.1.

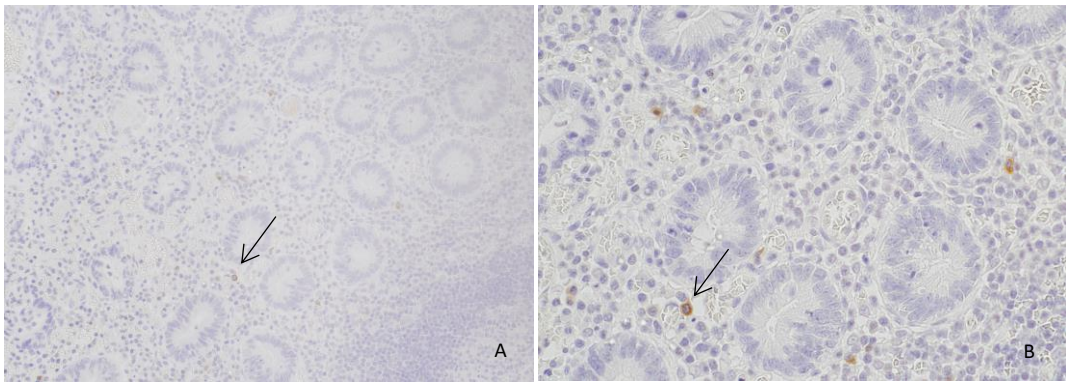


Figure 4.26: Expression of TNF-α (moderate) in monocytes of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 7.6.

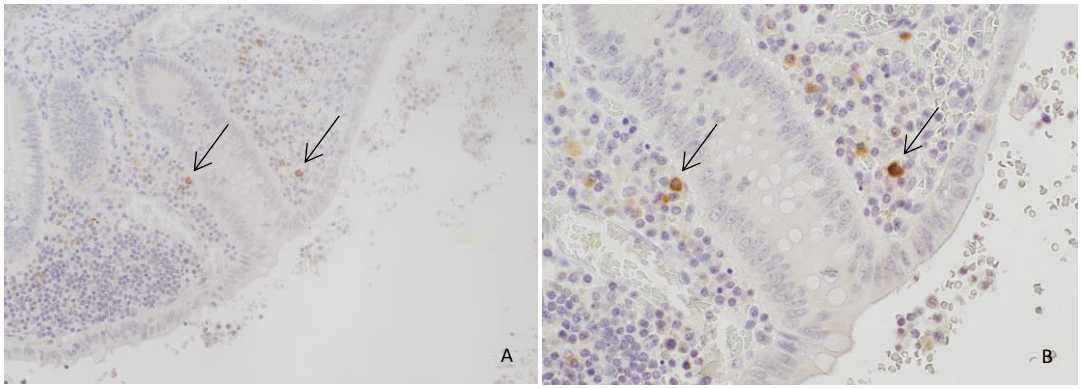


Figure 4.27: Expression of TNF-α (high) in monocytes of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 12.7.

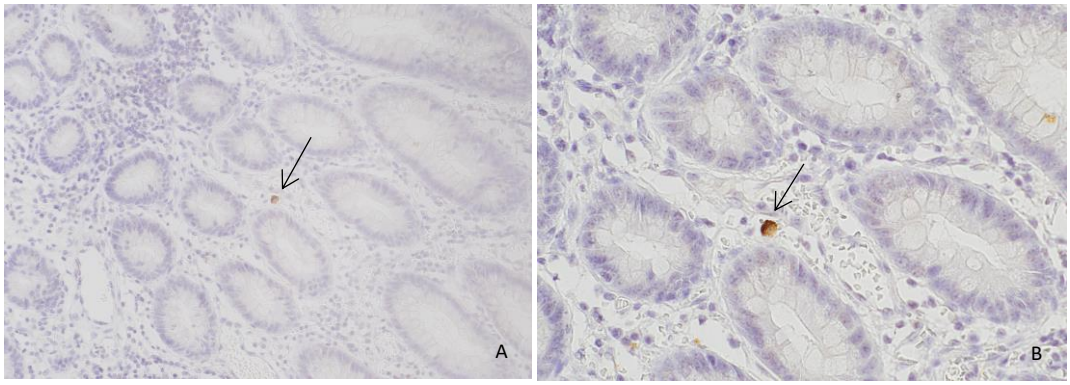


Figure 4.28: Expression of TNF-α (low) in monocytes of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 4.4.

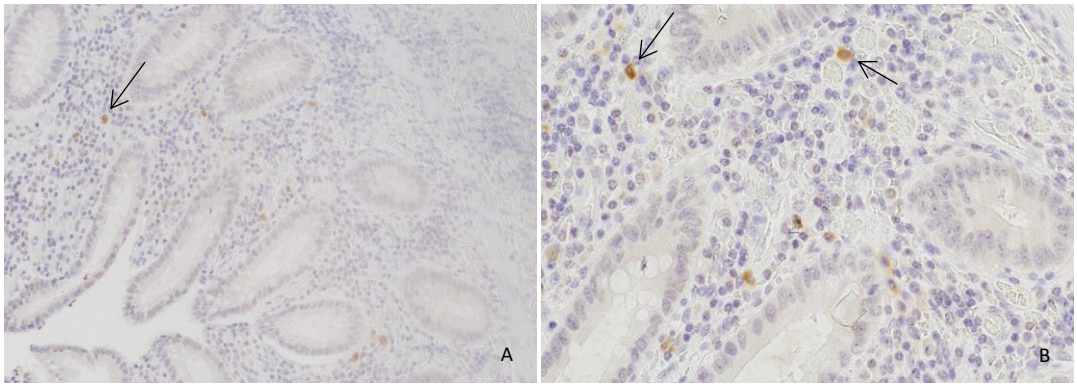


Figure 4.29: Expression of TNF-α (moderate) in monocytes of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 7.5.

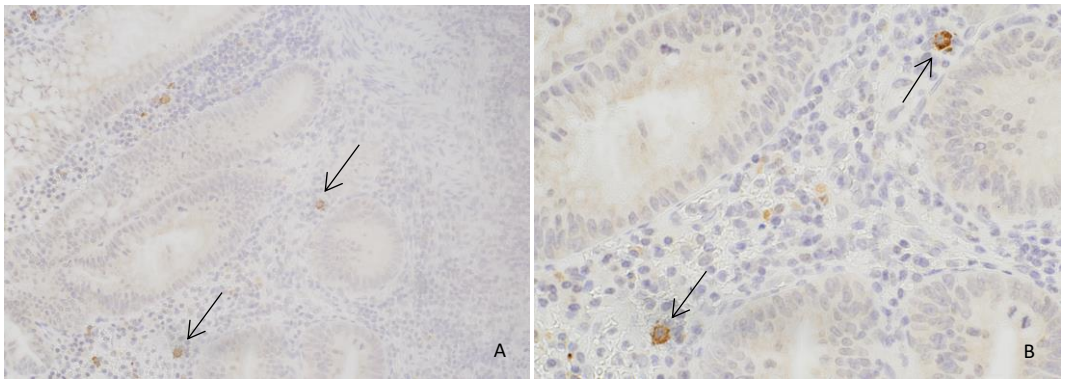


Figure 4.30: Expression of TNF-α (high) in monocytes of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 19.6.

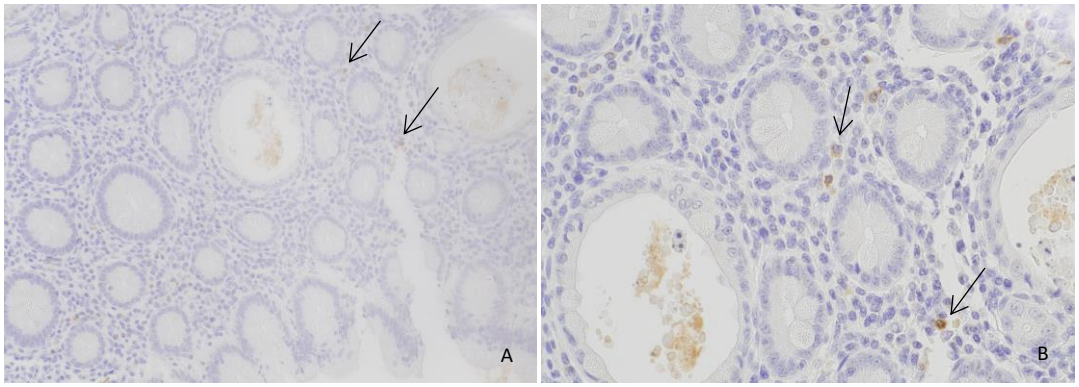


Figure 4.31: Expression of TNF-α (low) in monocytes of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 7.4.

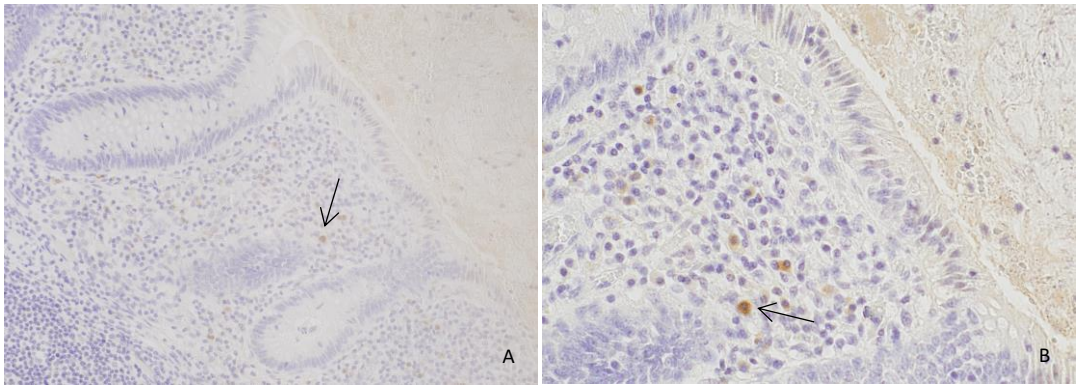


Figure 4.32: Expression of TNF-α (moderate) in monocytes of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 10.3.

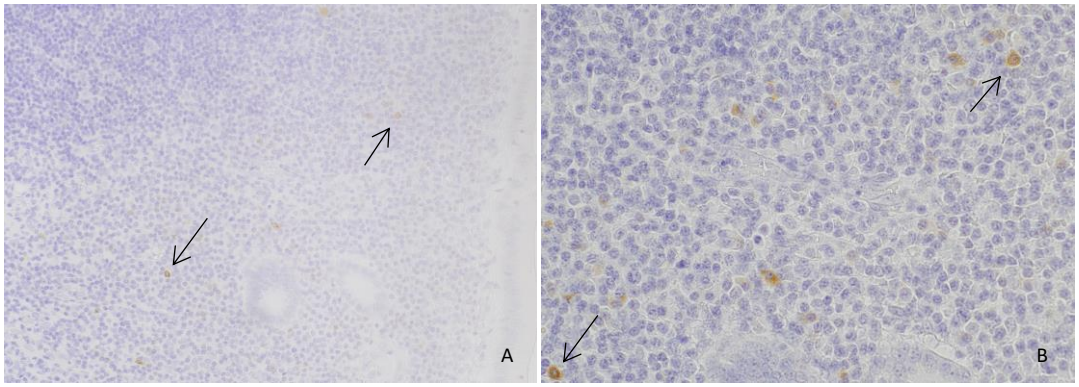


Figure 4.33: Expression of TNF-α (high) in monocytes of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 24.

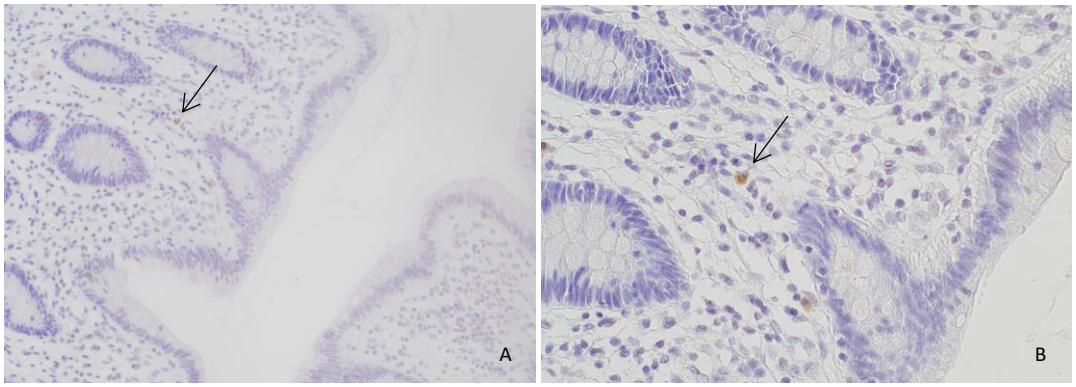


Figure 4.34: Expression of TNF-α (low) in monocytes of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 1.5.

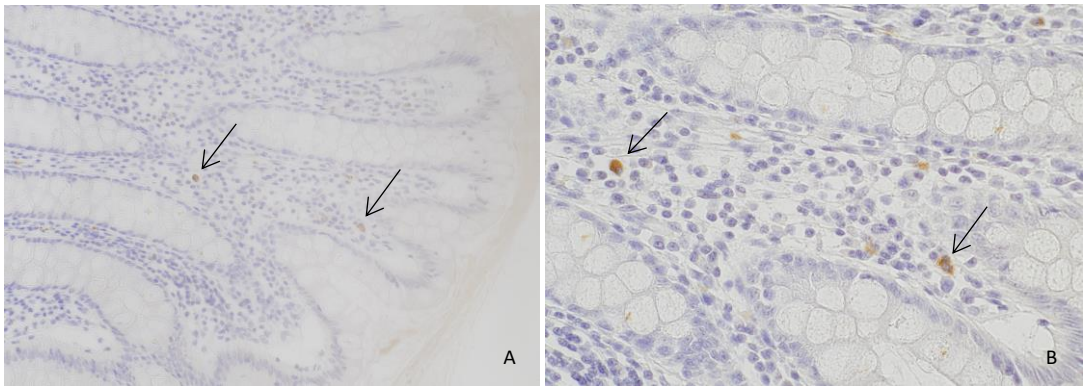


Figure 4.35: Expression of TNF-α (moderate) in monocytes of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 3.2.

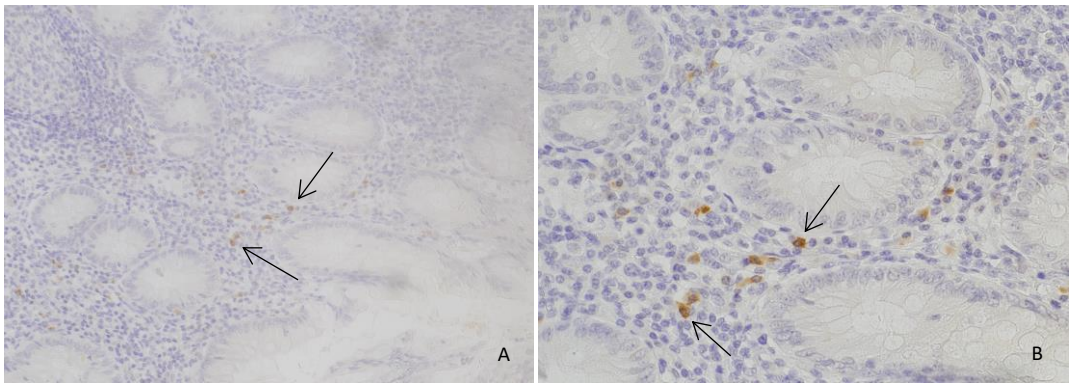


Figure 4.36: Expression of TNF-α (high) in monocytes of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to TNF-α (LSBio, LS-B7268) at a 7.5 µl/ml concentration for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The average number of brown cytoplasmic-stained cells in the mucosa (arrows), regardless of the intensity, was quantified. The score for this sample was 7.3.

4.2.3 Expression of IL-6

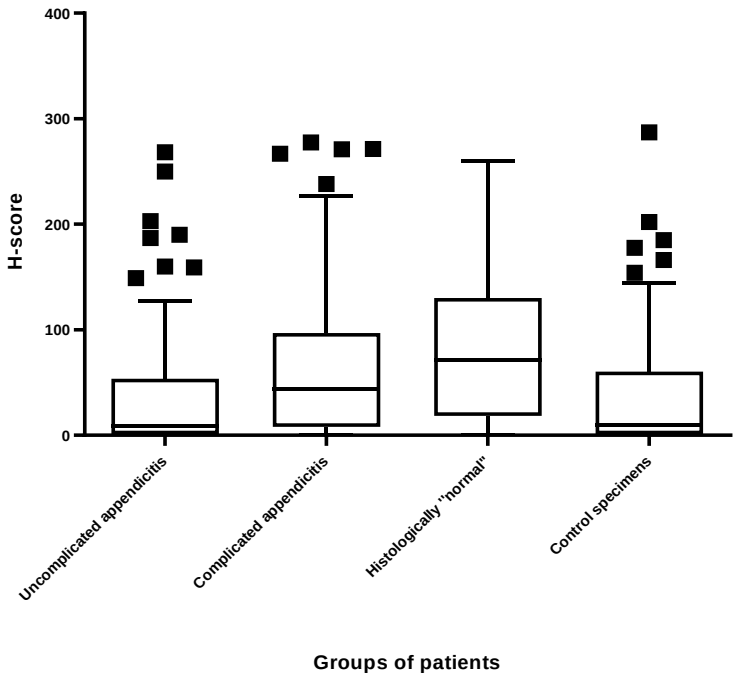
Immunohistochemistry performed with anti-IL-6 antibody revealed epithelial and inflammatory cells in the mucosa of the specimens. Regarding the inflammatory cells, staining was observed in both mononuclear and polymorphonuclear cells.

4.2.3.1 Expression of IL-6 in epithelial cells

The expression of IL-6 in epithelial cells was assessed on a total of 423 specimens as 25 samples did not have at least four high-power fields and therefore excluded from the study. Epithelial cells of specimens with complicated appendicitis (median 44.0, IQ range 8.0-97.0) demonstrated significantly increased expression of IL-6 compared with the control appendices (median 9.5, IQ range 1.0-60.25, $p<0.001$). The histologically "normal" appendices (median 71.0, IQ range 18.5-130.0) also showed significantly increased IL-6 expression compared with the control group (median 9.5, IQ range 1.0-60.25, $p<0.001$). However, IL-6 expression of acute uncomplicated appendicitis (median 9.0, IQ range 1.0-53.5) samples did not significantly differ from the control group (median 9.5, IQ range 1.0-60.2, $p=0.89$). Epithelial IL-6 expression was significantly higher in both group II (median 44.0, IQ range 8.0-97.0, $p<0.001$) and III (median 71.0, IQ range 18.5-130.0, $p<0.001$) compared with group I (median 9.0, IQ range 1.0-53.5). Moreover, histologically "normal" appendices (median 71.0, IQ range 18.5-130.0) showed significantly elevated levels of epithelial IL-6 expression compared with complicated appendicitis (median 44.0, IQ range 8.0-97.0, $p=0.04$) samples (Table 4.5, graph 4.5, figures 4.37-4.48).

Table 4.5: Summary of the analysis of IL-6 expression in epithelial cells between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	109	9.0	1.0	53.5	52.5	<0.001
II	107	44.0	8.0	97.0	89.0	
III	103	71.0	18.5	130.0	111.5	
IV	104	9.5	1.0	60.2	59.2	



Graph 4.5: Tukey's boxplots displaying data of IL-6 expression in epithelial cells between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 9.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 53.5, interquartile (IQ) range 52.5; Group II-complicated appendicitis specimens, median 44.0, 25th percentile (Q1) 8.0, 75th percentile (Q3) 97.0, interquartile (IQ) range 89.0; Group III-histologically "normal" specimens, median 71.0, 25th percentile (Q1) 18.5, 75th percentile (Q3) 130.0, interquartile (IQ) range 111.5; Group IV-control specimens, median 9.5, 25th percentile (Q1) 1.0, 75th percentile (Q3) 60.2, interquartile (IQ) range 59.2. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

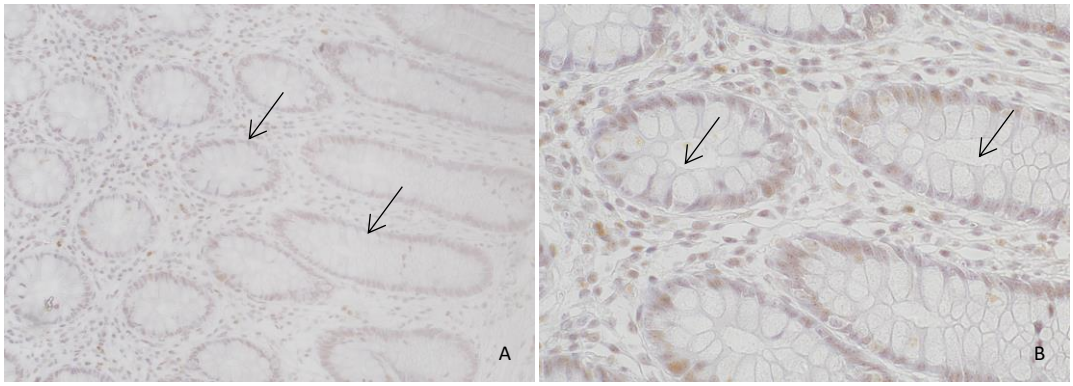


Figure 4.37: Expression of IL-6 (very low/negative) in epithelial cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The absence of brown cytoplasmic staining indicates no expression of IL-6 in the epithelial cells within the mucosa (arrows). The H-score for this sample was 0.

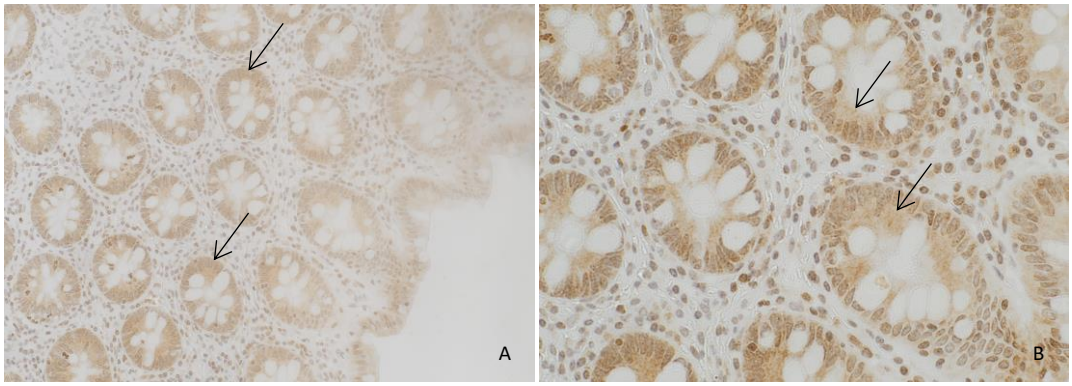


Figure 4.38: Expression of IL-6 (moderate) in epithelial cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The score for this sample was 16.

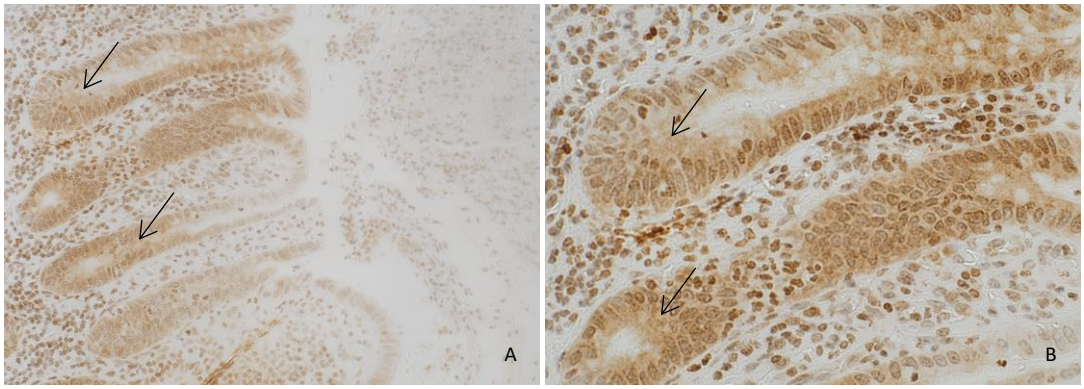


Figure 4.39: Expression of IL-6 (high) in epithelial cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 67.

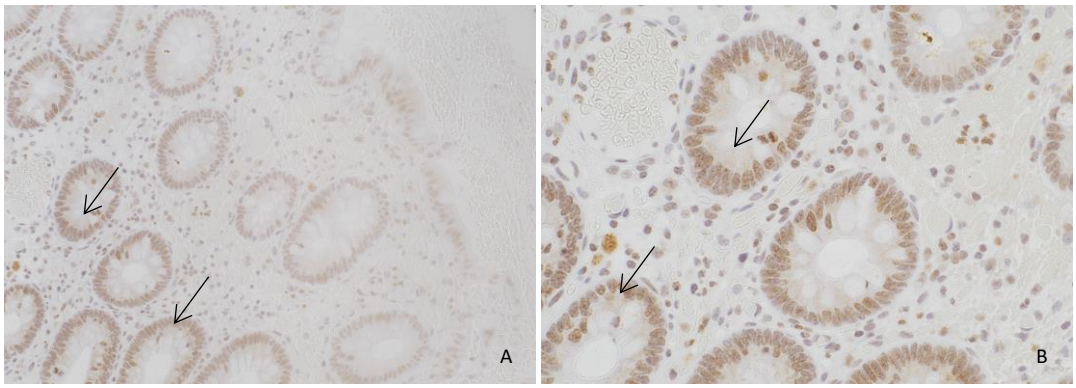


Figure 4.40: Expression of IL-6 (low) in epithelial cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 14.

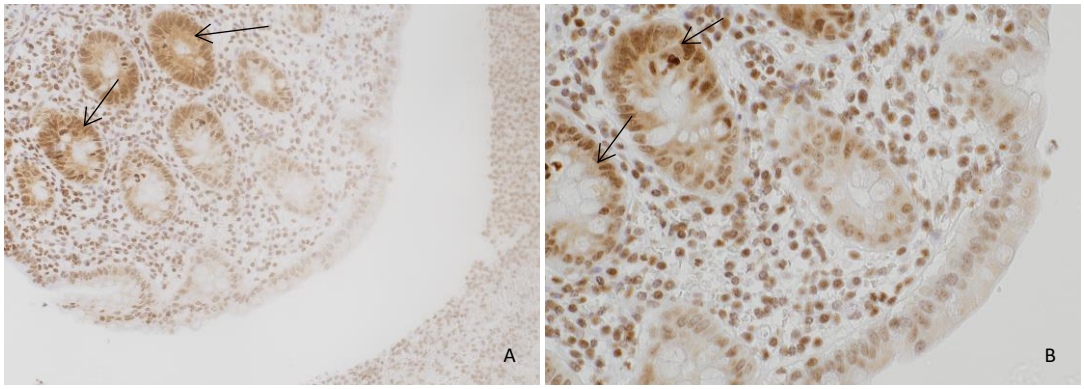


Figure 4.41: Expression of IL-6 (moderate to high) in epithelial cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 72.5.

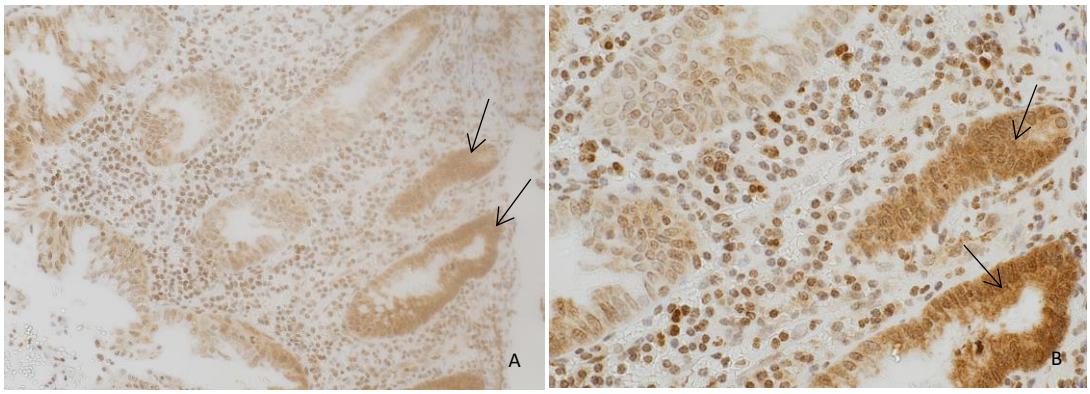


Figure 4.42: Expression of IL-6 (high) in epithelial cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 210.

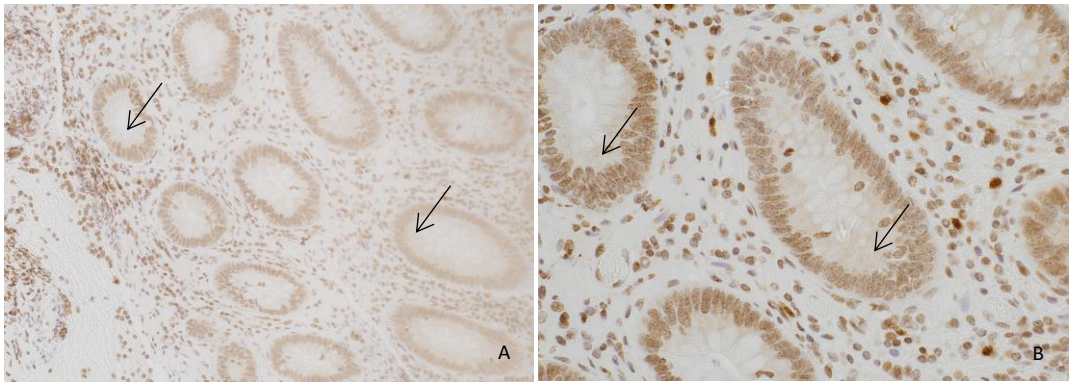


Figure 4.43: Expression of IL-6 (low) in epithelial cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 22.5.

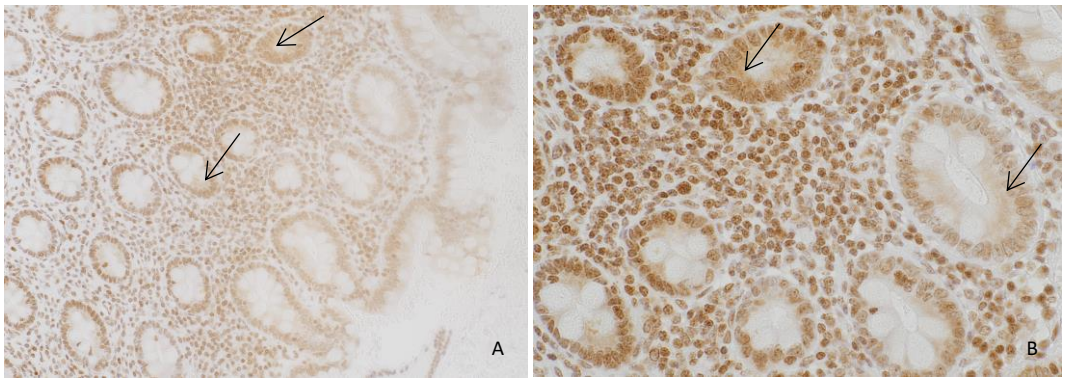


Figure 4.44: Expression of IL-6 (moderate) in epithelial cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 92.

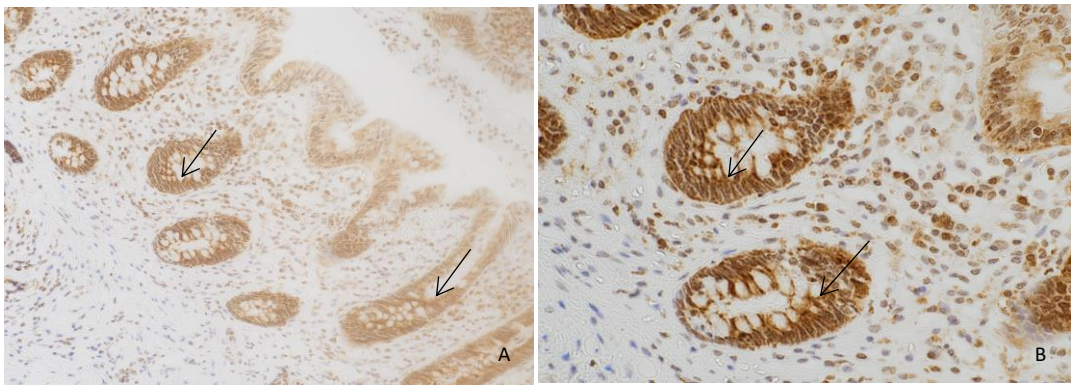


Figure 4.45: Expression of IL-6 (high) in epithelial cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 195.

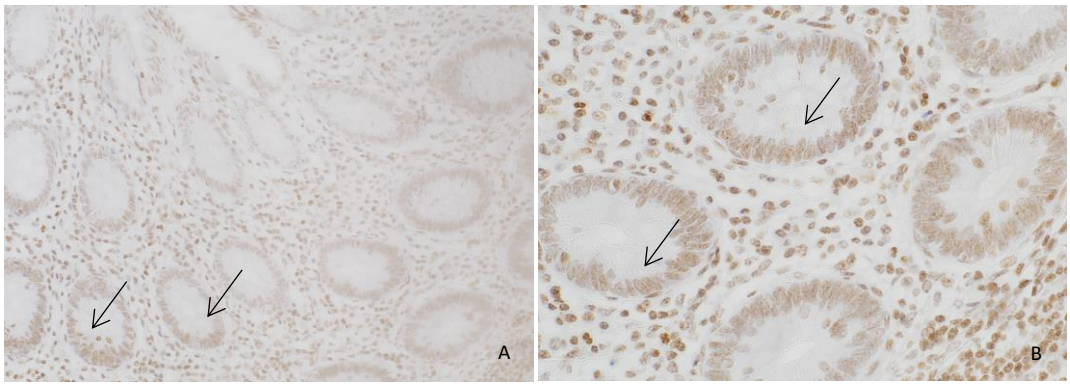


Figure 4.46: Expression of IL-6 (very low/negative) in epithelial cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The absence of brown cytoplasmic staining indicates no expression of IL-6 in the epithelial cells within the mucosa (arrows). The H-score for this sample was 0.

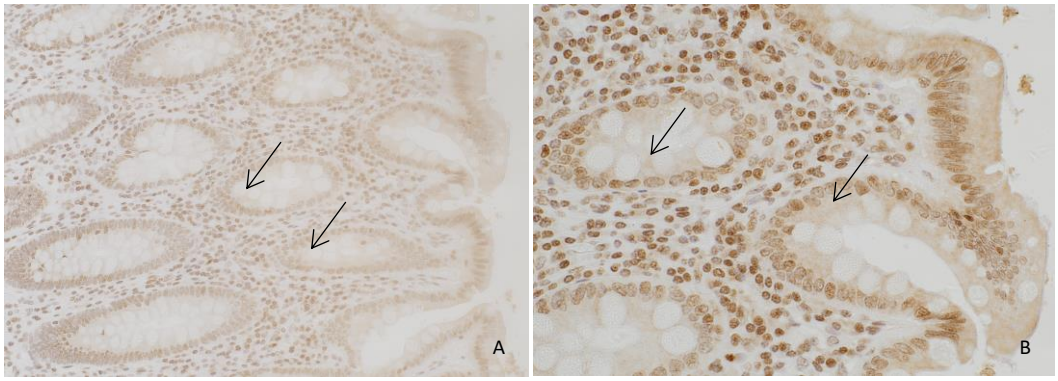


Figure 4.47: Expression of IL-6 (moderate) in epithelial cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 16.5.

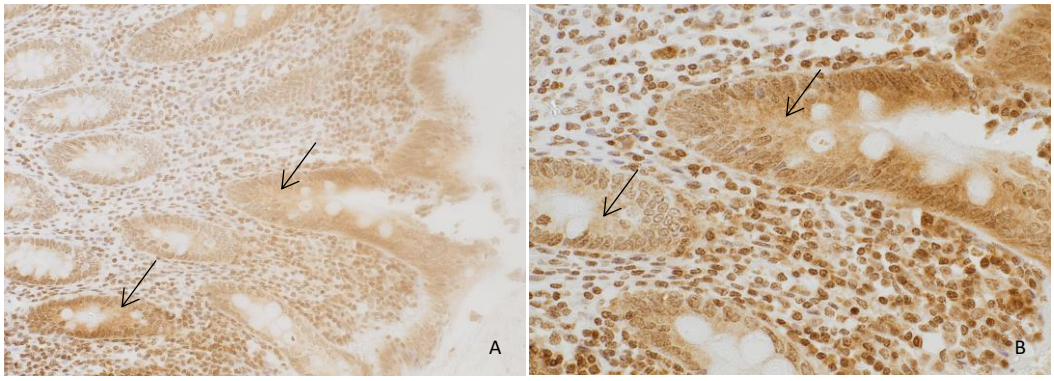


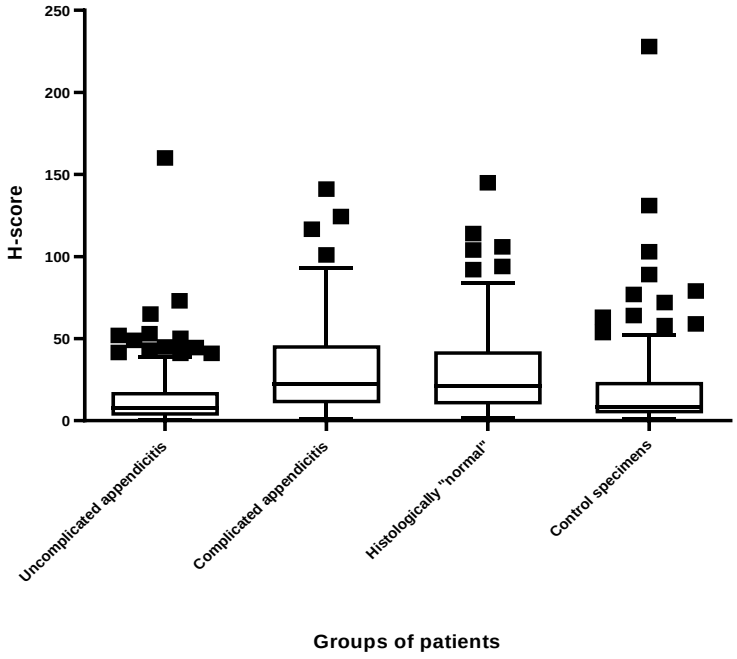
Figure 4.48: Expression of IL-6 (high) in epithelial cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained epithelial cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the epithelium. The positive brown cytoplasmic staining indicates epithelial cells within the glands of the mucosa (arrows). The H-score for this sample was 88.

4.2.3.2 Expression of IL-6 in inflammatory cells

The expression of IL-6 in inflammatory cells was assessed on a total of 423 specimens as 25 samples did not have at least four high-power fields and therefore excluded from the study. Complicated appendicitis (median 22.5. IQ range 10.6-46.0) group demonstrated significantly increased expression of IL-6 secreted by inflammatory cells compared with the control group (median 8.3, IQ range 4.4-23.6, $p<0.001$). Histologically "normal" appendices (median 21.0, IQ range 10.0-42.2) also demonstrated significantly elevated expression of IL-6 compare with the control group (median 8.3, IQ range 4.4-23.6, $p<0.001$). However, IL-6 expression of acute uncomplicated appendicitis (median 8.0, IQ range 3.0-17.5) samples did not significantly differ from the control group (median 8.3, IQ range 4.4-23.6, $p=0.07$). Groups II (median 22.5. IQ range 10.6-46.0, $p<0.001$) and III (median 21.0, IQ range 10.0-42.2, $p<0.001$) demonstrated significantly increased inflammatory cell IL-6 expression compared with group I (median 8.0, IQ range 3.0-17.5). Moreover, the inflammatory cell expression of IL-6 did not differ significantly between the groups of histologically "normal" (median 21.0, IQ range 10.0-42.2) and complicated appendicitis (median 22.5. IQ range 10.6-46.0, $p=0.63$) samples (Table 4.6, graph 4.6, figures 4.49-4.60).

Table 4.6: Summary of the analysis of IL-6 expression in inflammatory cells between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	109	8.0	3.0	17.5	14.5	<0.001
II	107	22.5	10.6	46.0	35.3	
III	103	21.0	10.0	42.2	32.2	
IV	104	8.3	4.4	23.6	19.2	



Graph 4.6: Tukey's boxplots displaying data of IL-6 expression in inflammatory cells between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 8.0, 25th percentile (Q1) 3.0, 75th percentile (Q3) 17.5, interquartile (IQ) range 14.5; Group II-complicated appendicitis specimens, median 22.5, 25th percentile (Q1) 10.6, 75th percentile (Q3) 46.0, interquartile (IQ) range 35.3; Group III-histologically "normal" specimens, median 21.0, 25th percentile (Q1) 10.0, 75th percentile (Q3) 42.2, interquartile (IQ) range 32.2; Group IV-control specimens, median 8.3, 25th percentile (Q1) 4.4, 75th percentile (Q3) 23.6, interquartile (IQ) range 19.2. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

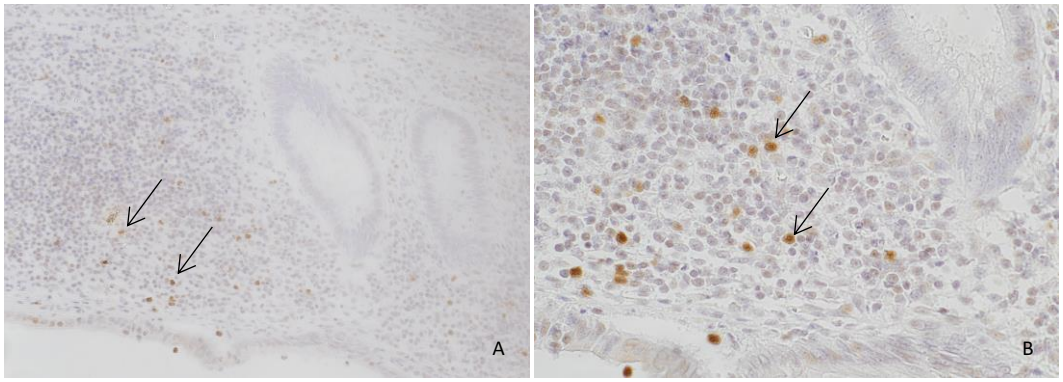


Figure 4.49: Expression of IL-6 (low) in inflammatory cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 1.2.

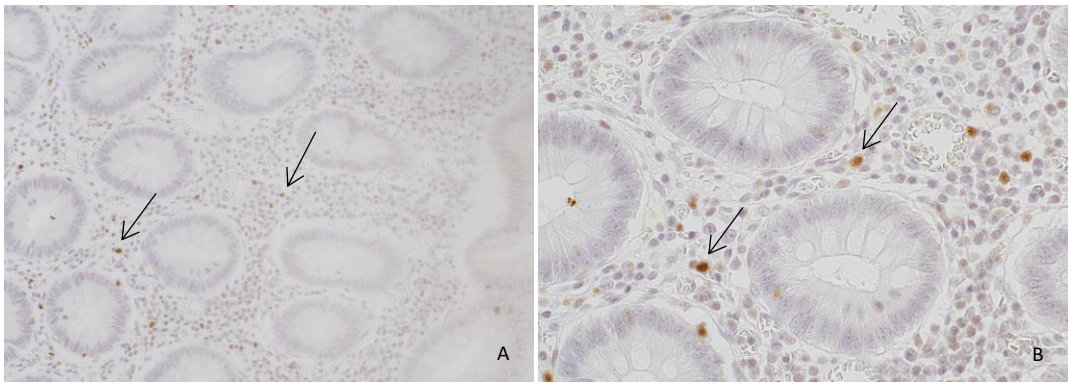


Figure 4.50: Expression of IL-6 (moderate) in inflammatory cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 9.1.

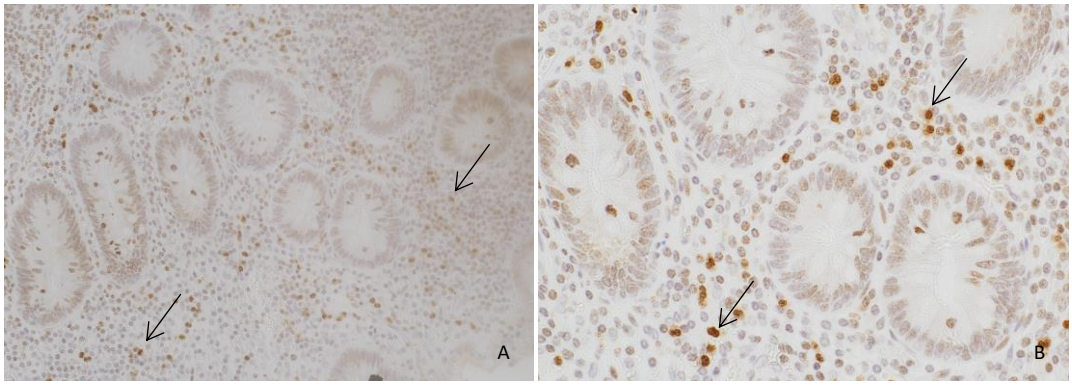


Figure 4.51: Expression of IL-6 (high) in inflammatory cells of a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 27.5.

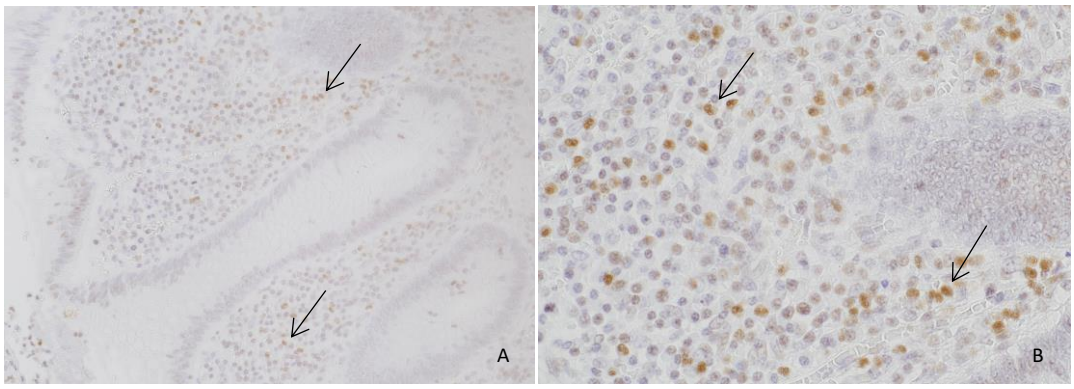


Figure 4.52: Expression of IL-6 (low) in inflammatory cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 10.

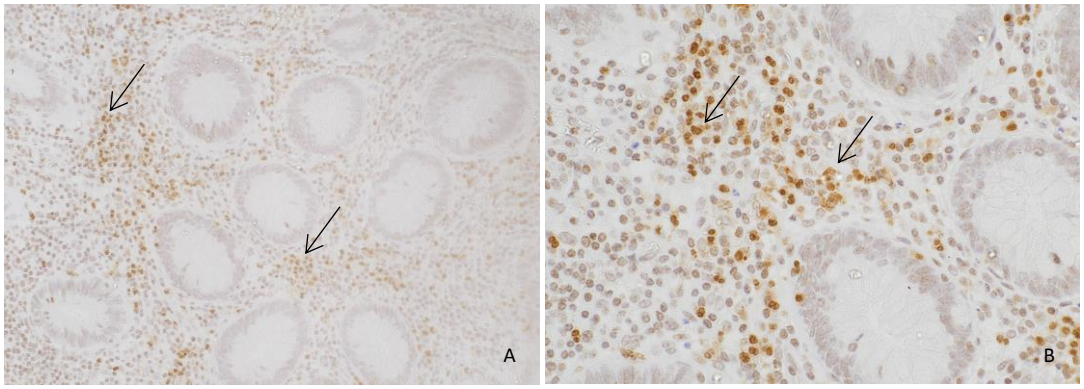


Figure 4.53: Expression of IL-6 (moderate) in inflammatory cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 29.

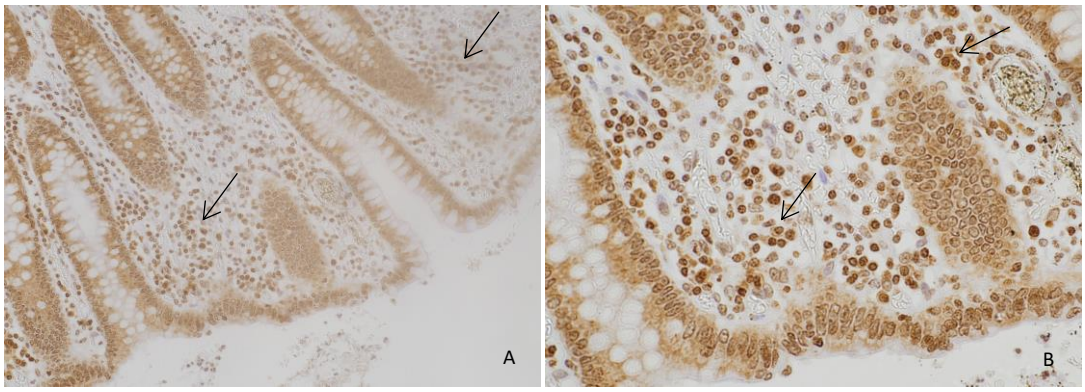


Figure 4.54: Expression of IL-6 (high) in inflammatory cells of a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 83.

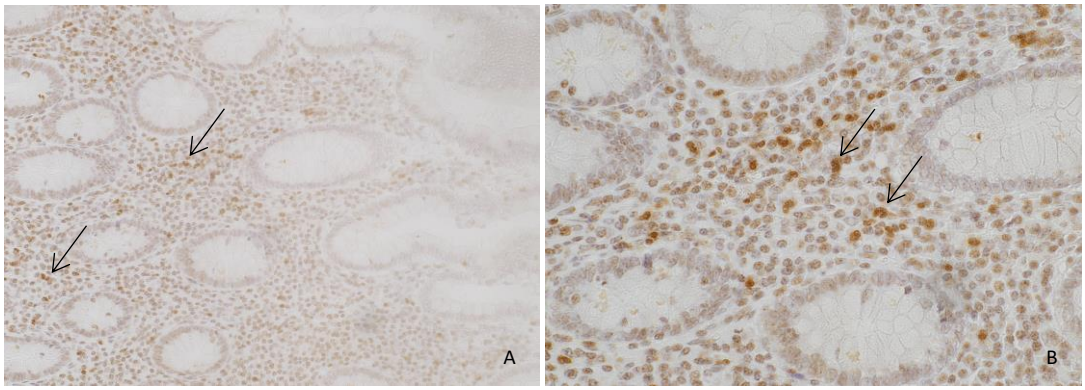


Figure 4.55: Expression of IL-6 (low to moderate) in inflammatory cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 13.9.

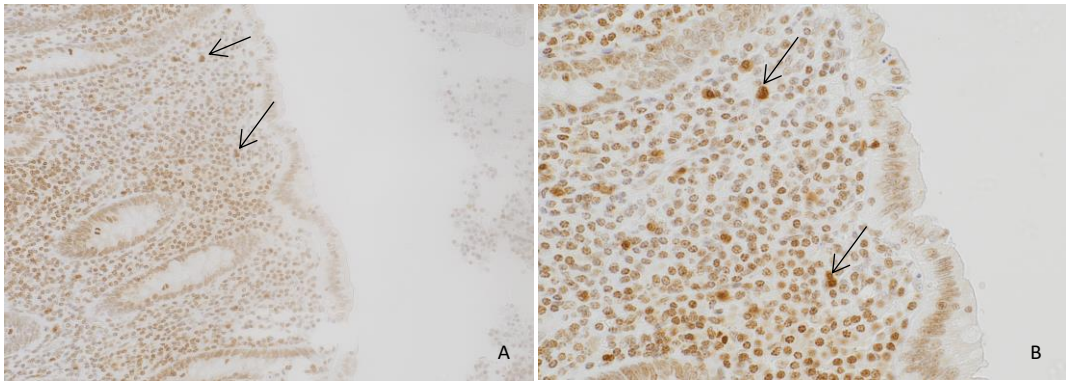


Figure 4.56: Expression of IL-6 (moderate) in inflammatory cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 20.

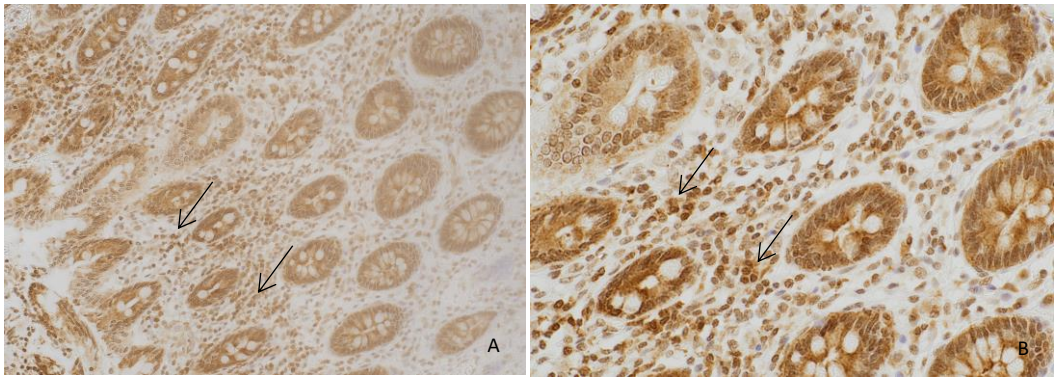


Figure 4.57: Expression of IL-6 (high) in inflammatory cells of a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 114.

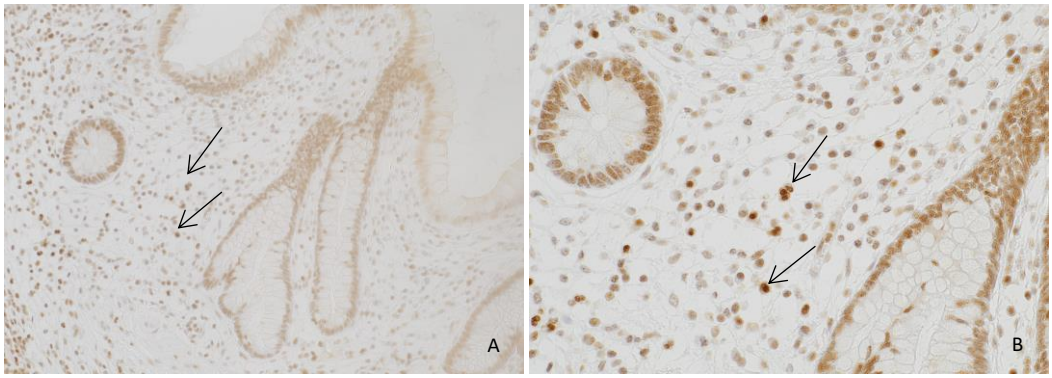


Figure 4.58: Expression of IL-6 (low) in inflammatory cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 4.

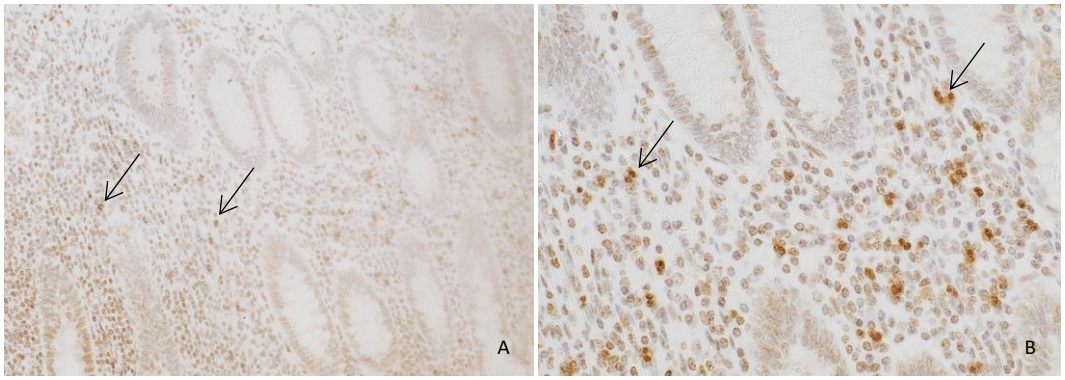


Figure 4.59: Expression of IL-6 (moderate) in inflammatory cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 10.4.

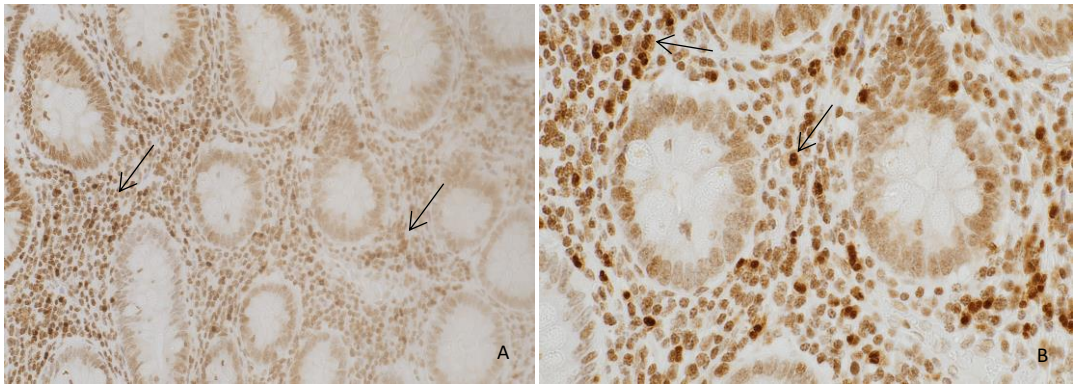


Figure 4.60: Expression of IL-6 (high) in inflammatory cells of a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-6 (Leica, NCL-L-IL6) at a 1:50 dilution for 60 mins at RT. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The H-score [% of positive cells (brown cytoplasmic-stained inflammatory cells) × intensity of staining (1 to 3)] was used to assess the expression of IL-6 in the inflammatory cells. The positive brown cytoplasmic staining indicates inflammatory cells within the mucosa (arrows). The H-score for this sample was 58.

4.2.4 Expression of IL-2R

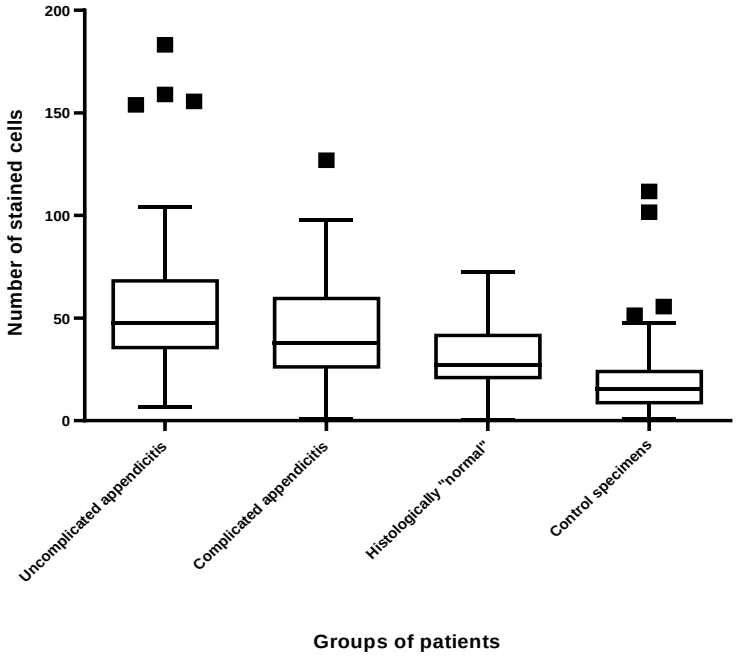
Immunohistochemistry performed with anti-IL-2R revealed lymphocytes in the mucosa and submucosa of the samples. In the germinal centres IL-2R expression was extremely intense making it impossible to be quantified. Therefore, quantification of IL-2R expression in the submucosa was performed between the germinal centres.

4.2.4.1 Expression of IL-2R in mucosa

The expression of IL-2R in mucosa was assessed on a total of 417 specimens as 31 samples did not have at least three high-power fields and therefore excluded from the study. Mucosal IL-2R expression was significantly increased in both groups of acute uncomplicated (median 47.4, IQ 34.8-69.0, $p<0.001$) and complicated (median 37.8, IQ range 25.4-60.4, $p<0.001$) appendicitis compared with the control group (median 15,4, IQ range 7.9-24.8), as was IL-2R expression in the histologically "normal" appendices (median 27.0, IQ range 20.2-42.4) compared with the control specimens (median 15,4, IQ range 7.9-24.8, $p<0.001$). Both group I (median 47.4, IQ 34.8-69.0, $p<0.001$) and II (median 37.8, IQ range 25.4-60.4, $p<0.001$) demonstrated significantly increased IL-2R expression compared with group III (median 27.0, IQ range 20.2-42.4). Moreover, IL-2R expression was significantly higher in the acute uncomplicated appendicitis (median 47.4, IQ 34.8-69.0) samples compared with the complicated appendicitis (median 37.8, IQ range 25.4-60.4, $p=0.001$) samples (Table 4.7, graph 4.7, figures 4.61-4.72).

Table 4.7: Summary of the analysis of IL-2R expression in mucosa between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	109	47.4	34.8	69.0	34.2	<0.001
II	103	37.8	25.4	60.4	35.0	
III	103	27.0	20.2	42.4	22.2	
IV	102	15.4	7.9	24.8	16.8	



Graph 4.7: Tukey's boxplots displaying data of IL-2R expression in the mucosa between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 47.4, 25th percentile (Q1) 34.8, 75th percentile (Q3) 69.0, interquartile (IQ) range 34.2; Group II-complicated appendicitis specimens, median 37.8, 25th percentile (Q1) 25.4, 75th percentile (Q3) 60.4, interquartile (IQ) range 35.0; Group III-histologically "normal" specimens, median 27.0, 25th percentile (Q1) 20.2, 75th percentile (Q3) 42.4, interquartile (IQ) range 22.2; Group IV-control specimens, median 15.4, 25th percentile (Q1) 7.9, 75th percentile (Q3) 24.8, interquartile (IQ) range 16.8. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

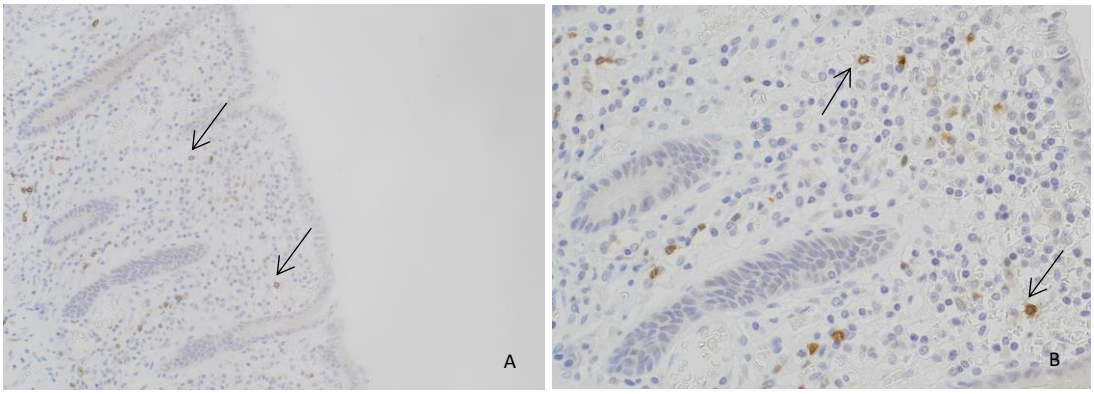


Figure 4.61: Expression of IL-2R (low) in inflammatory cells of the mucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 29.8.

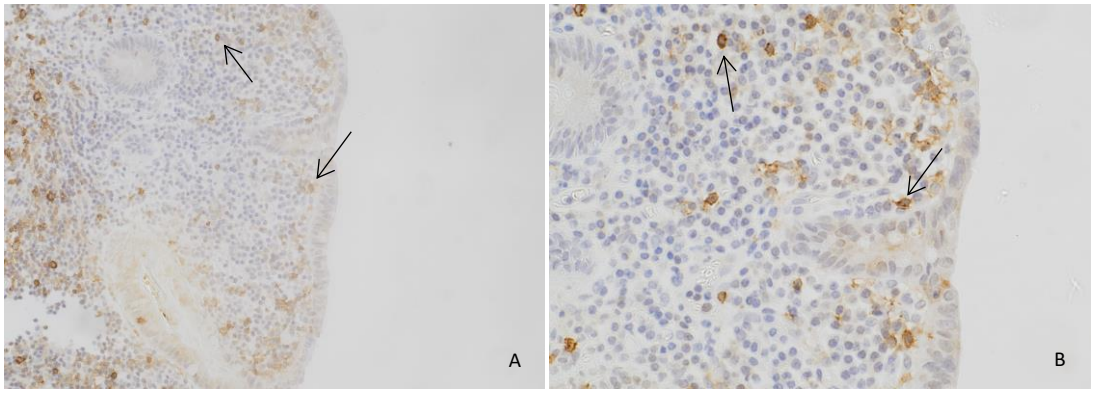


Figure 4.62: Expression of IL-2R (moderate) in inflammatory cells of the mucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 55.4.

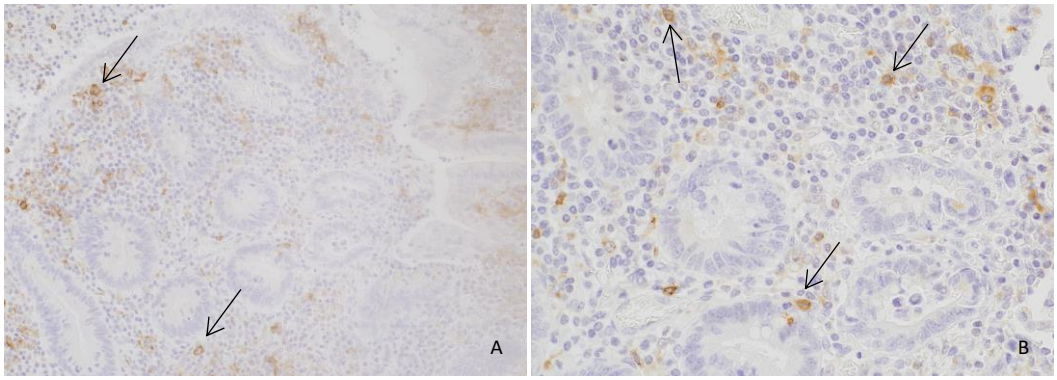


Figure 4.63: Expression of IL-2R (high) in inflammatory cells of the mucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 76.8.

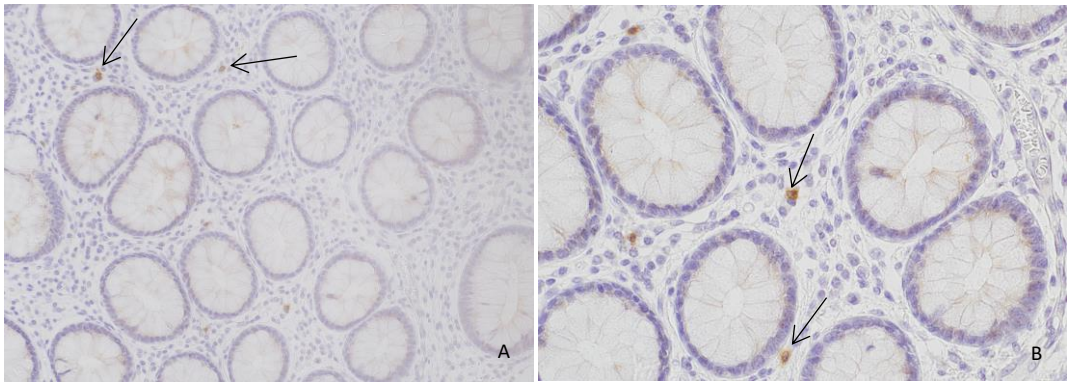


Figure 4.64: Expression of IL-2R (low) in inflammatory cells of the mucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 16.4.

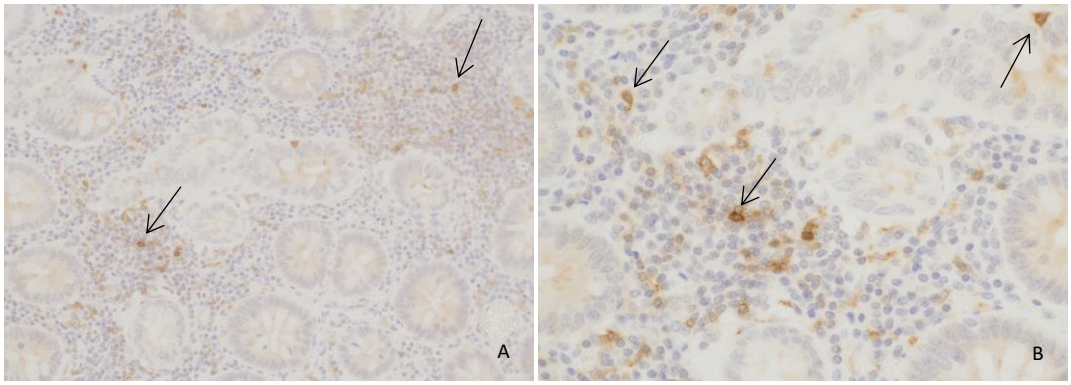


Figure 4.65: Expression of IL-2R (moderate) in inflammatory cells of the mucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 38.6.

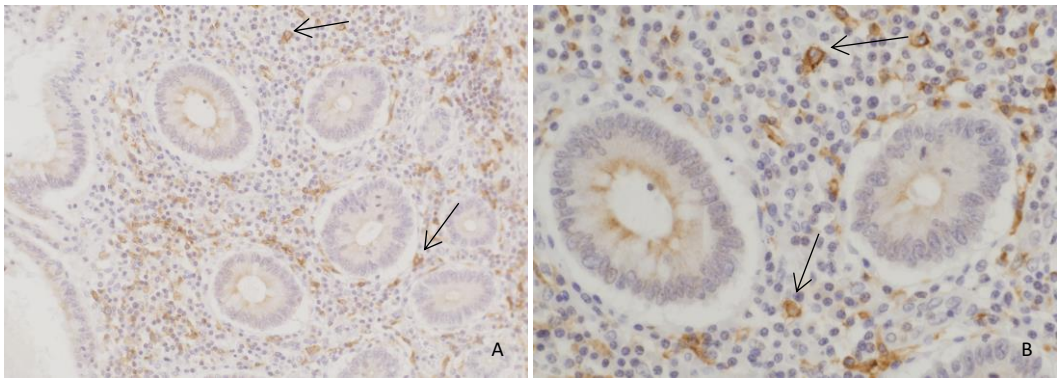


Figure 4.66: Expression of IL-2R (high) in inflammatory cells of the mucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 84.

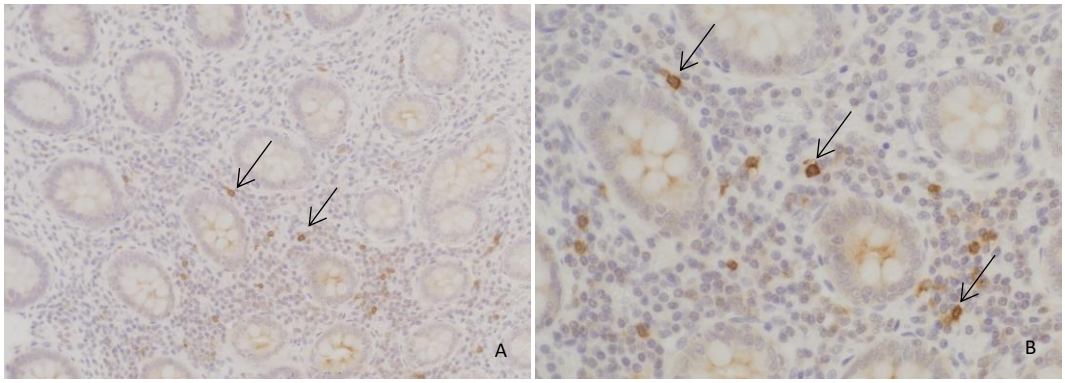


Figure 4.67: Expression of IL-2R (low) in inflammatory cells of the mucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 14.8.

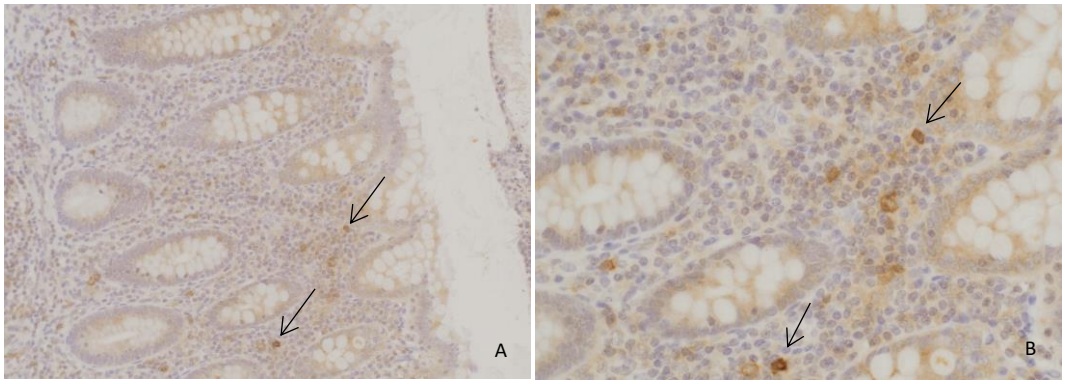


Figure 4.68: Expression of IL-2R (moderate) in inflammatory cells of the mucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 28.

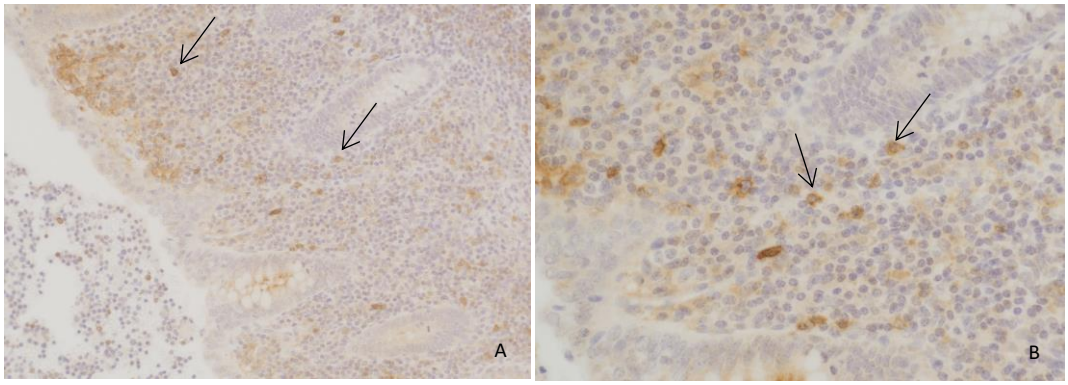


Figure 4.69: Expression of IL-2R (high) in inflammatory cells of the mucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 52.

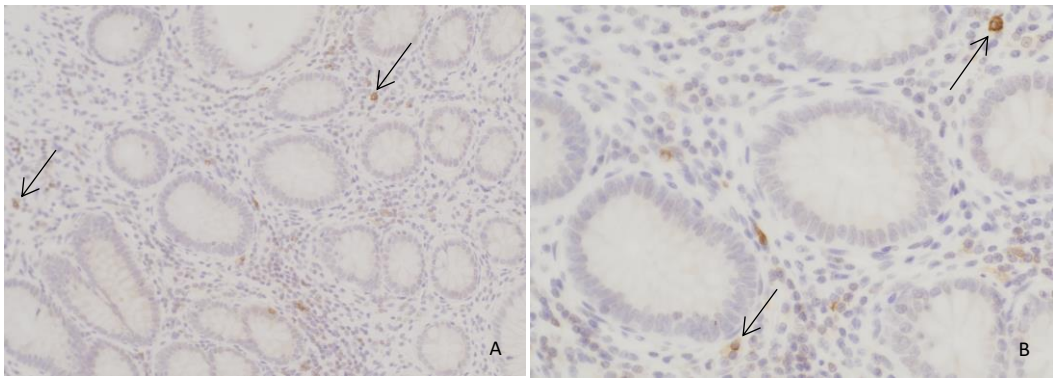


Figure 4.70: Expression of IL-2R (low) in inflammatory cells of the mucosa in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa, regardless of the intensity (arrows), was counted. The score for this sample was 5.

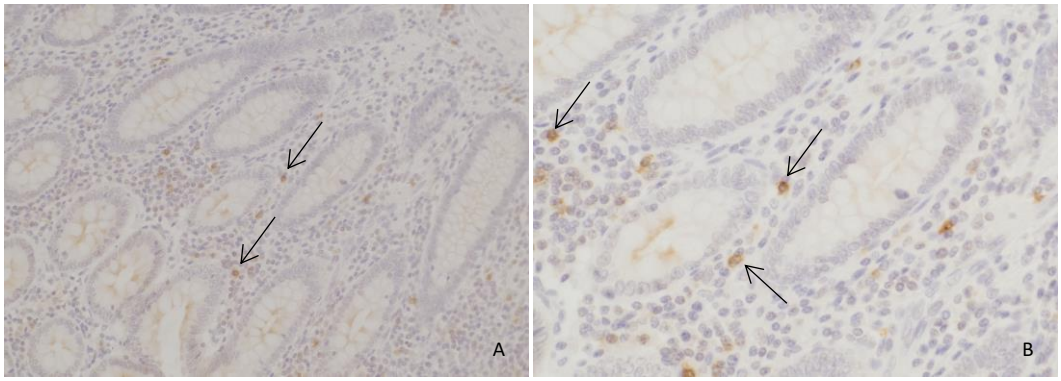


Figure 4.71: Expression of IL-2R (moderate) in inflammatory cells of the mucosa in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa (arrows), regardless of the intensity, was counted. The score for this sample was 15.8.

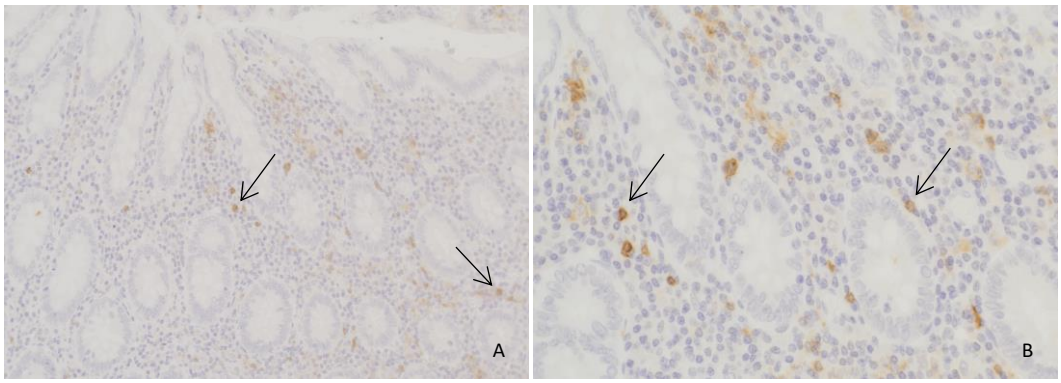


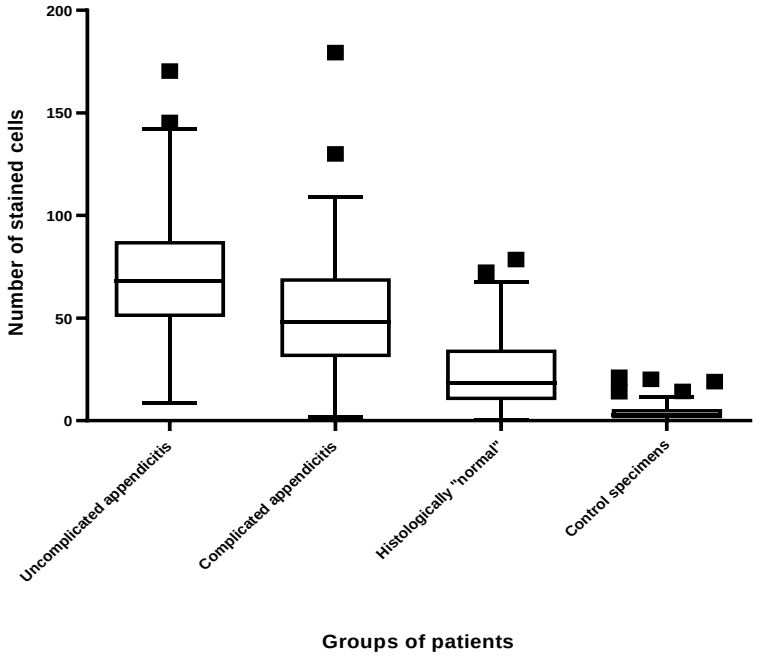
Figure 4.72: Expression of IL-2R (high) in inflammatory cells of the mucosa in a patient from the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the mucosa, regardless of the intensity (arrows), was counted. The score for this sample was 30.4.

4.2.4.2 Expression of IL-2R in submucosa

The expression of IL-2R in submucosa was assessed on 445 specimens as 3 samples did not have at least three high-power fields and therefore excluded from the study. Both groups of acute uncomplicated (median 67.8, IQ range 50.5-87.6, $p<0.001$) and complicated (median 47.9, IQ range 30.9-69.4, $p<0.001$) appendicitis demonstrated significantly increased IL-2R expression in the submucosa compared with the control group (median 2.8, IQ range 1.2-5.7). The histologically "normal" appendices (median 18.4, IQ range 10.1-34.7) also expressed significantly increased IL-2R expression compared with the control group (median 2.8, IQ range 1.2-5.7, $p<0.001$). Moreover, both group I (median 67.8, IQ range 50.5-87.6, $p<0.001$) and group II (median 47.9, IQ range 30.9-69.4, $p<0.001$) showed significantly increased expression of IL-2R in the submucosa compared with group III (median 18.4, IQ range 10.1-34.7). In a similar pattern to mucosal expression, IL-2R expression of the acute uncomplicated appendicitis group (median 67.8, IQ range 50.5-87.6) was found to be significantly increased compared with the complicated appendicitis (median 47.9, IQ range 30.9-69.4, $p<0.001$) samples (Table 4.8, graph 4.8, figures 4.73-4.84).

Table 4.8: Summary of the analysis of IL-2R expression in submucosa between the 4 groups of specimens. Group I-specimens with uncomplicated appendicitis, Group II-specimens with complicated appendicitis, Group III-histologically "normal" appendices, Group IV-control specimens. N represents the number of specimens that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	120	67.8	50.5	87.6	37.0	<0.001
II	118	47.9	30.9	69.4	38.5	
III	104	18.4	10.1	34.7	24.6	
IV	103	2.8	1.2	5.7	4.5	



Graph 4.8: Tukey's boxplots displaying data of IL-2R expression in the submucosa between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 67.8, 25th percentile (Q1) 50.5, 75th percentile (Q3) 87.6, interquartile (IQ) range 37.0; Group II-complicated appendicitis specimens, median 47.9, 25th percentile (Q1) 30.9, 75th percentile (Q3) 69.4, interquartile (IQ) range 38.5; Group III-histologically "normal" specimens, median 18.4, 25th percentile (Q1) 10.1, 75th percentile (Q3) 34.7, interquartile (IQ) range 24.6; Group IV-control specimens, median 2.8, 25th percentile (Q1) 1.2, 75th percentile (Q3) 5.7, interquartile (IQ) range 4.5. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

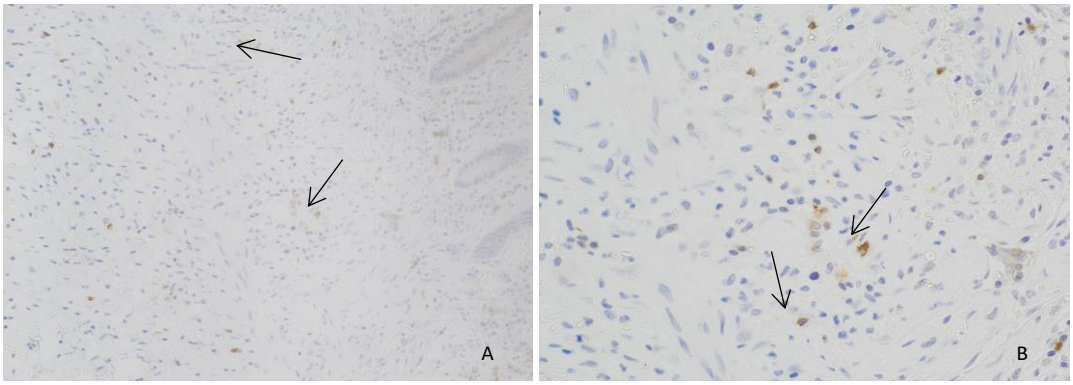


Figure 4.73: Expression of IL-2R (low) in inflammatory cells of the submucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 39.6.

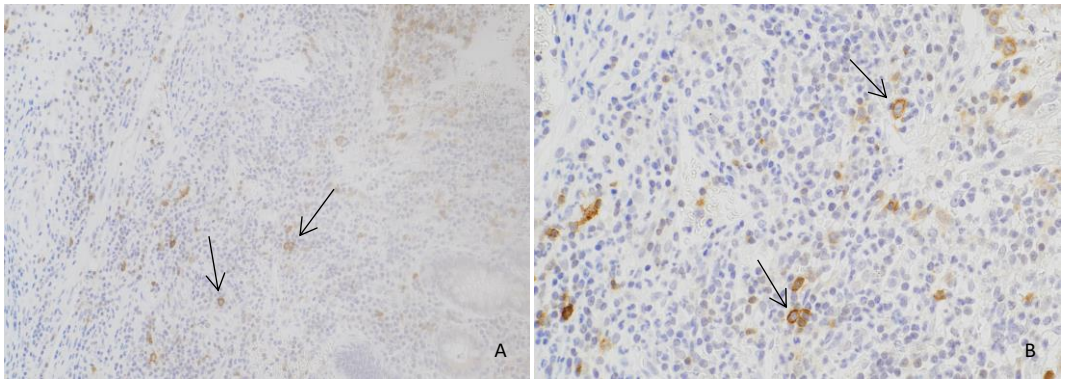


Figure 4.74: Expression of IL-2R (moderate) in inflammatory cells of the submucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 65.4.

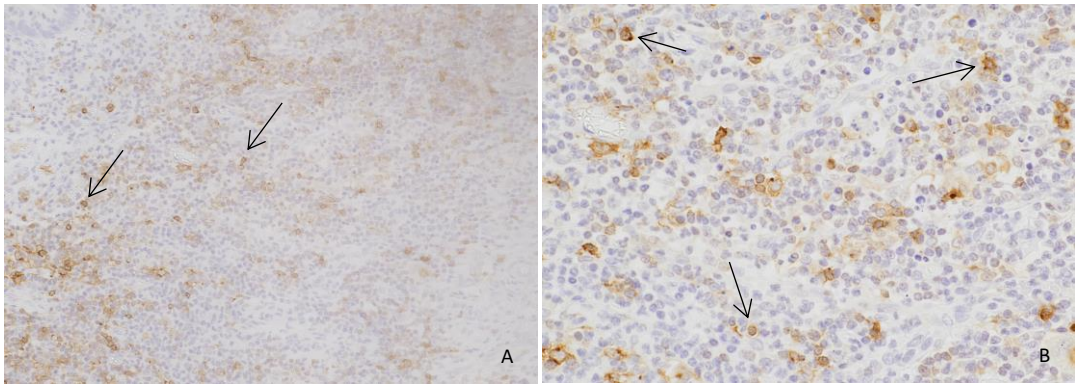


Figure 4.75: Expression of IL-2R (high) in inflammatory cells of the submucosa in a patient with acute uncomplicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 101.8.

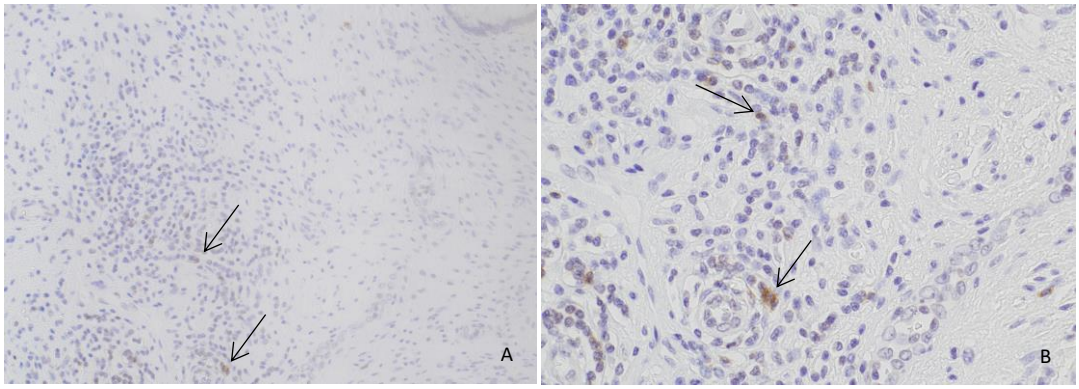


Figure 4.76: Expression of IL-2R (low) in inflammatory cells of the submucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 9.4.

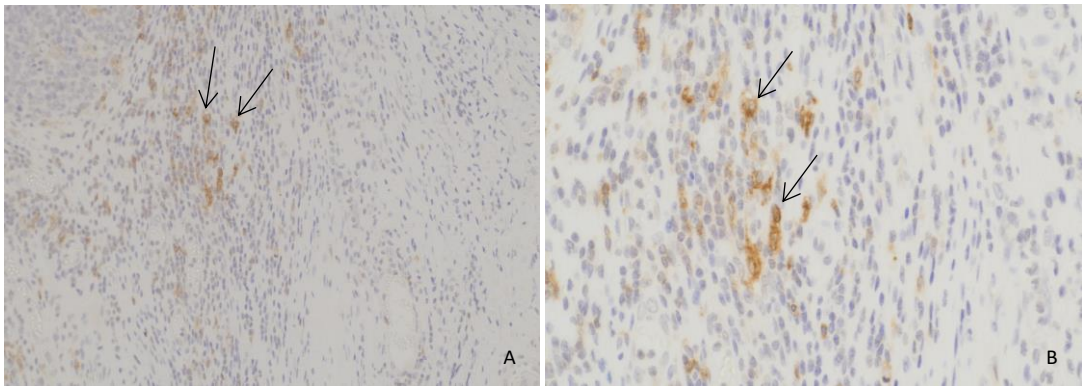


Figure 4.77: Expression of IL-2R (moderate) in inflammatory cells of the submucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 47.4.

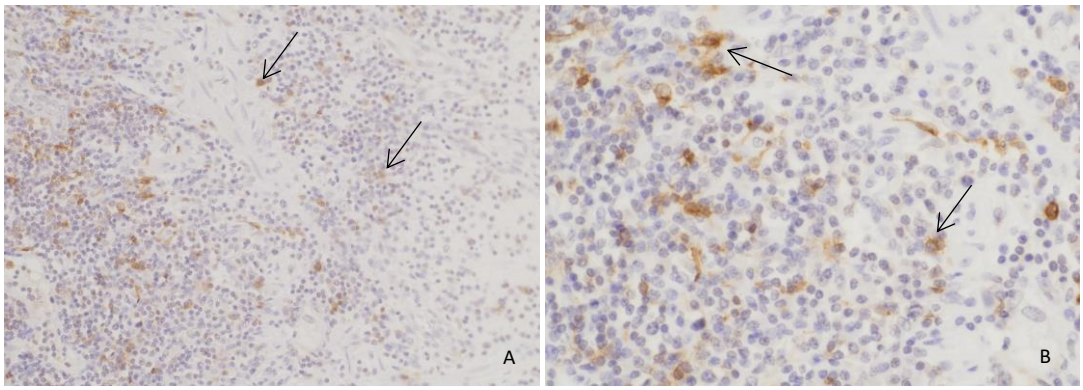


Figure 4.78: Expression of IL-2R (high) in inflammatory cells of the submucosa in a patient with acute complicated appendicitis, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 85.6.

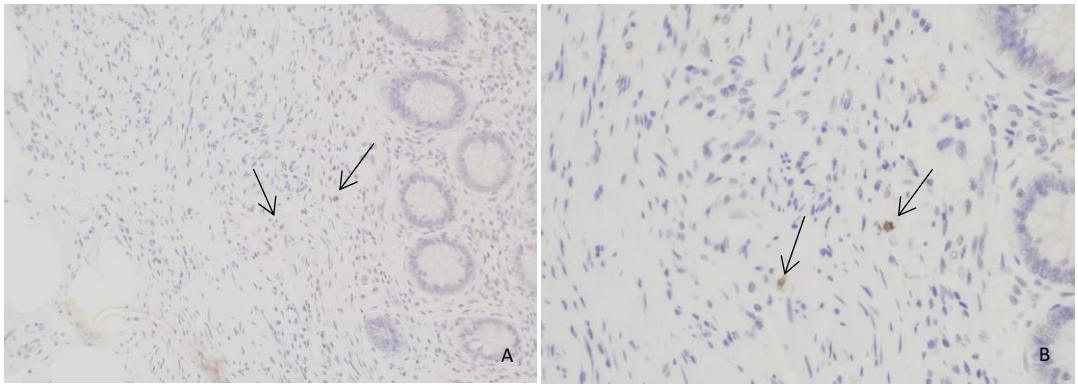


Figure 4.79: Expression of IL-2R (low) in inflammatory cells of the submucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 7.

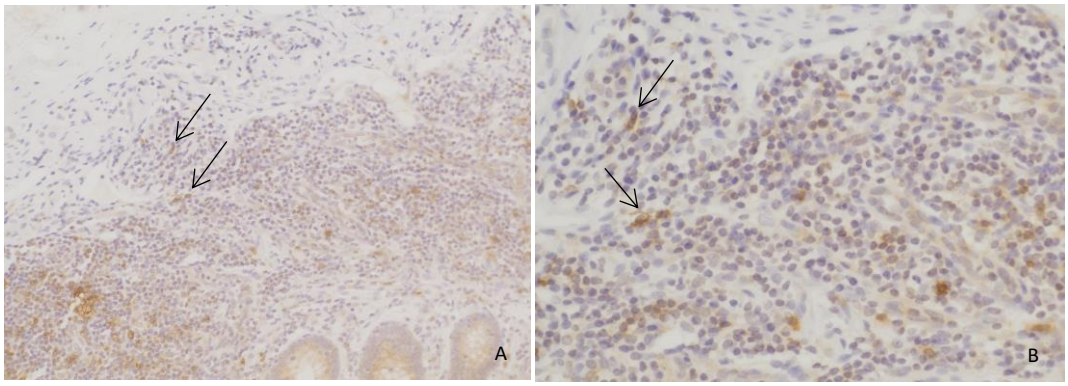


Figure 4.80: Expression of IL-2R (moderate) in inflammatory cells of the submucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 18.2.

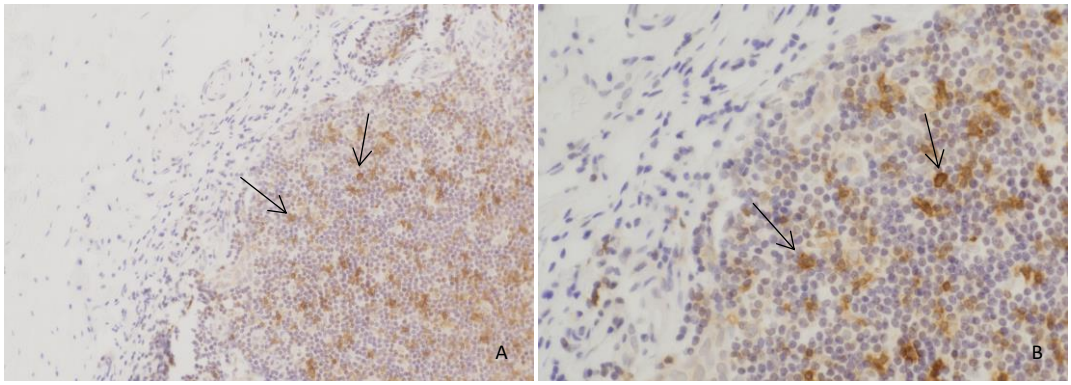


Figure 4.81: Expression of IL-2R (high) in inflammatory cells of the submucosa in a patient with a histologically "normal" appendix, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 47.2.

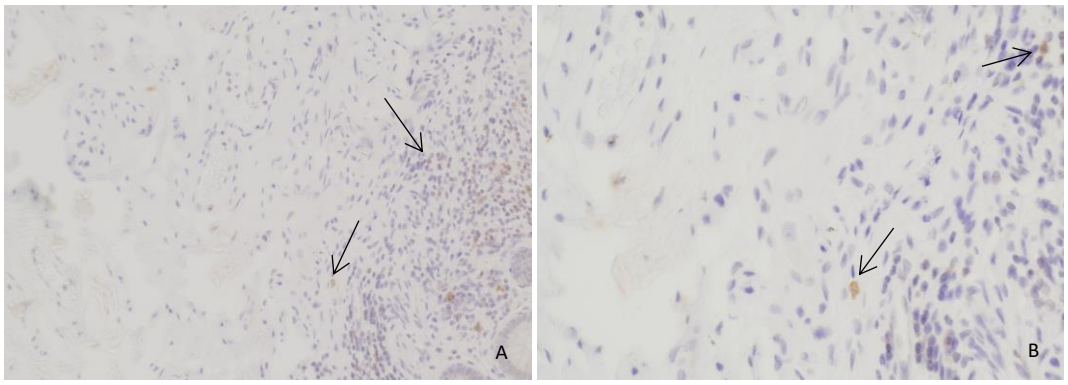


Figure 4.82: Expression of IL-2R (low) in inflammatory cells of the submucosa in a patient of the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 0.8.

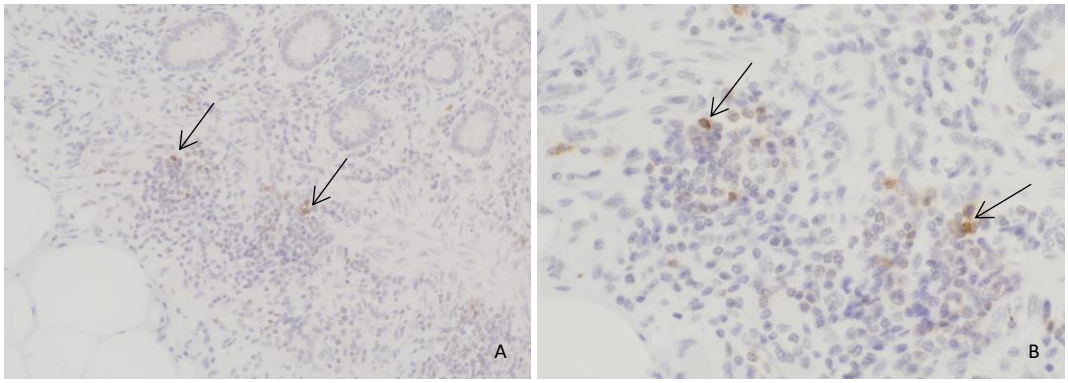


Figure 4.83: Expression of IL-2R (moderate) in inflammatory cells of the submucosa in a patient of the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 3.2.

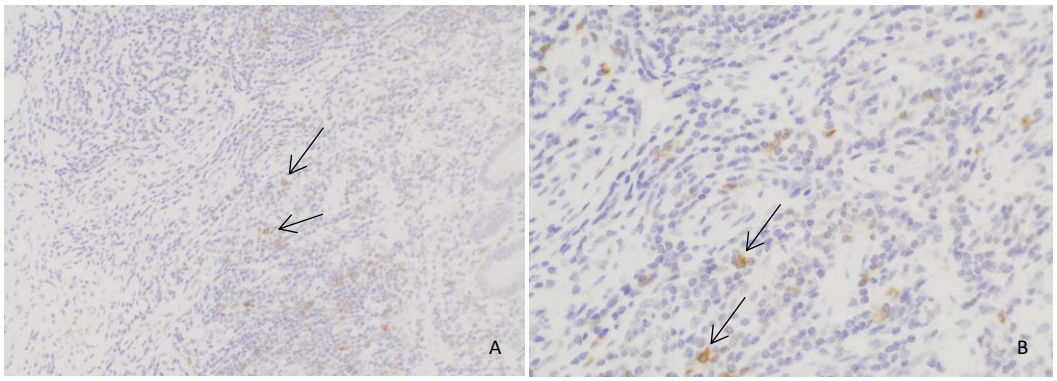


Figure 4.84: Expression of IL-2R (high) in inflammatory cells of the submucosa in a patient of the control group, using IHC staining, at ×200 (A) and ×400 (B) magnification. Heat-mediated antigen retrieval was performed using citrate buffer, pH 6 (20 mins). The sections were then incubated with monoclonal antibody to IL-2R (Leica, NCL-CD25-305) at a 1:400 dilution for overnight at 4 °C. Polymeric HRP-linker antibody conjugate was used as secondary antibody. DAB chromogen was used to visualise the staining. The sections were counterstained with haematoxylin. The number of brown membrane-stained cells in the submucosa (arrows), regardless of the intensity, was counted. The score for this sample was 6.6.

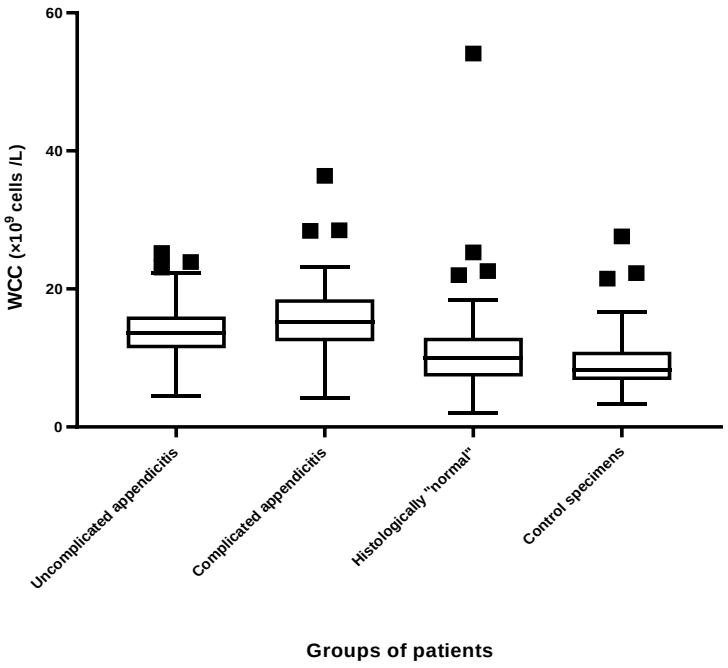
4.3 Clinical data

4.3.1 White cell count

The white cell count (WCC) was assessed on 436 patients as data was not available on 12 patients. Both groups of patients with acute uncomplicated (median 13.6, IQ range 11.4-16.0, $p<0.001$) and complicated (median 15.2, IQ range 12.4-18.5, $p<0.001$) appendicitis demonstrated significantly increased WCC compared with the control group (median 8.3, IQ range 6.8-10.9). Patients with histologically "normal" appendices (median 10.0, IQ range 7.3-12.6) also demonstrated significantly increased WCC compared with the control group (median 8.3, IQ range 6.8-10.9, $p=0.02$). Moreover, both group I (median 13.6, IQ range 11.4-16.0, $p<0.001$) and group II (median 15.2, IQ range 12.4-18.5, $p<0.001$) showed significantly increased WCC compared with group III (median 10.0, IQ range 7.3-12.6). Patients with complicated (median 15.2, IQ range 12.4-18.5) appendicitis demonstrated a significantly higher WCC compared with patients with acute uncomplicated (median 13.6, IQ range 11.4-16.0, $p=0.007$) appendicitis (Table 4.9, graph 4.9).

Table 4.9: Summary of the analysis of the WCC between the 4 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	119	13.6	11.4	16.0	4.6	<0.001
II	118	15.2	12.4	18.5	6.0	
III	104	10.0	7.3	12.6	5.6	
IV	95	8.3	6.8	10.9	4.1	



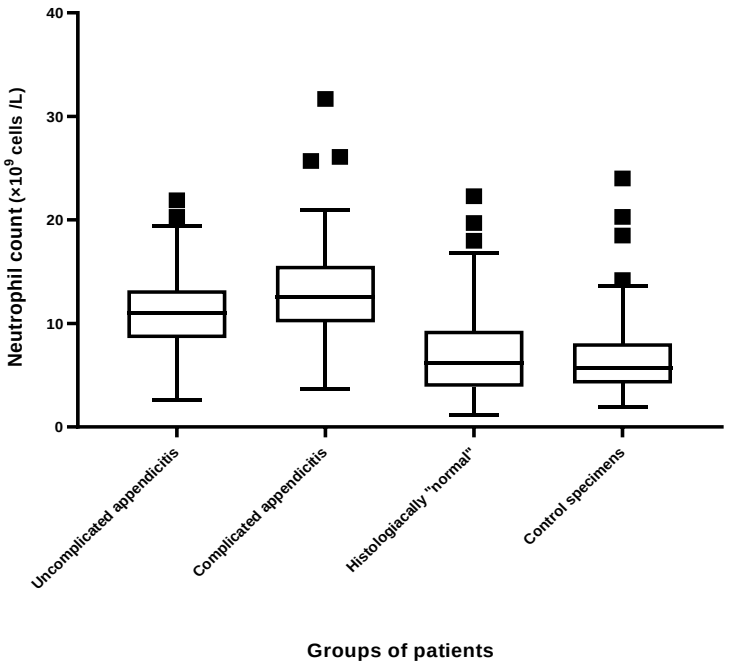
Graph 4.9: Tukey's boxplots displaying data of the WCC between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 13.6, 25th percentile (Q1) 11.4, 75th percentile (Q3) 16.0, interquartile (IQ) range 4.6; Group II-complicated appendicitis specimens, median 15.2, 25th percentile (Q1) 12.4, 75th percentile (Q3) 18.5, interquartile (IQ) range 6.0; Group III-histologically "normal" specimens, median 10.0, 25th percentile (Q1) 7.3, 75th percentile (Q3) 12.6, interquartile (IQ) range 5.6; Group IV-control specimens, median 8.3, 25th percentile (Q1) 6.8, 75th percentile (Q3) 10.9, interquartile (IQ) range 4.1. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.2 Neutrophil count

The neutrophil count was assessed on 436 patients as data was not available on 12 patients. Both groups of patients with acute uncomplicated (median 11.0, IQ range 8.6-13.2, p<0.001) and complicated (median 12.6, IQ range 10.1-15.5, p<0.001) appendicitis demonstrated significantly increased neutrophil count compared with the control group (median 5.7, IQ range 4.2-9.3). Patients with histologically "normal" appendices (median 6.2, IQ range 3.9-8.1) did not demonstrate significant difference in the neutrophil count compared with the control group (median 5.7, IQ range 4.2-9.3, p=0.50). Both group I (median 11.0, IQ range 8.6-13.2, p<0.001) and group II (median 12.6, IQ range 10.1-15.5, p<0.001) showed significantly increased neutrophil count compared with group III (median 6.2, IQ range 3.9-8.1). Moreover, patients with complicated (median 12.6, IQ range 10.1-15.5) appendicitis demonstrated a higher neutrophil count compared with patients with acute uncomplicated (median 11.0, IQ range 8.6-13.2, p=0.002) appendicitis (Table 4.10, graph 4.10).

Table 4.10: Summary of the analysis of the neutrophil count between the 4 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	119	11.0	8.6	13.2	4.6	<0.001
II	118	12.6	10.1	15.5	5.4	
III	104	6.2	3.9	8.1	5.4	
IV	95	5.7	4.2	9.3	3.9	



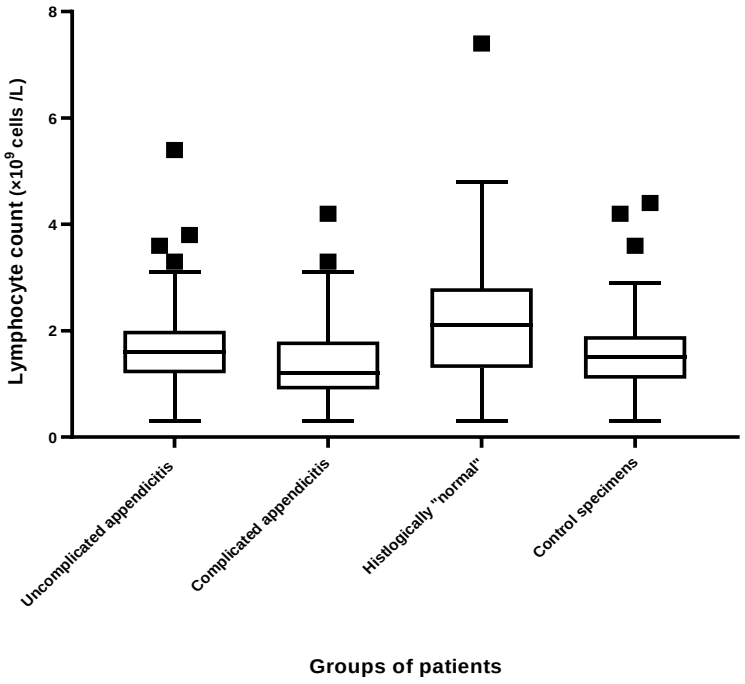
Graph 4.10: Tukey's boxplots displaying data of the neutrophil count between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 11.0, 25th percentile (Q1) 8.6, 75th percentile (Q3) 13.2, interquartile (IQ) range 4.6; Group II-complicated appendicitis specimens, median 12.6, 25th percentile (Q1) 10.1, 75th percentile (Q3) 15.5, interquartile (IQ) range 5.4; Group III-histologically "normal" specimens, median 6.2, 25th percentile (Q1) 3.9, 75th percentile (Q3) 8.1, interquartile (IQ) range 5.4; Group IV-control specimens, median 5.7, 25th percentile (Q1) 4.2, 75th percentile (Q3) 9.3, interquartile (IQ) range 3.9. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.3 Lymphocyte count

The lymphocyte count was assessed on 435 patients as data was not available on 13 patients. Patients with acute uncomplicated (median 1.6, IQ range 1.2-2.0) appendicitis did not demonstrate significant difference in the lymphocyte count compared with the control group (median 1.5, IQ range 1.1-1.9, p=0.44). Patients with complicated (median 1.2, IQ range 0.9-1.8) appendicitis demonstrated significantly reduced lymphocyte count compared with the control group (median 1.5, IQ range 1.1-1.9, p=0.01). Patients with histologically "normal" appendices (median 2.1, IQ range 1.3-2.8) demonstrated significantly increased lymphocyte count compared with the control group (median 1.5, IQ range 1.1-1.9, p<0.001) as well as with group I (median 1.6, IQ range 1.2-2.0, p<0.001) and group II (median 1.2, IQ range 0.9-1.8, p<0.001). Moreover, patients with acute uncomplicated (median 1.6, IQ range 1.2-2.0) appendicitis demonstrated significantly higher lymphocyte count compared with patients complicated (median 1.2, IQ range 0.9-1.8, p=0.002) appendicitis (Table 4.11, graph 4.11).

Table 4.11: Summary of the analysis of the lymphocyte count between the 4 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	119	1.6	1.2	2.0	0.8	<0.001
II	118	1.2	0.9	1.8	0.9	
III	103	2.1	1.3	2.8	1.5	
IV	95	1.5	1.1	1.9	0.8	



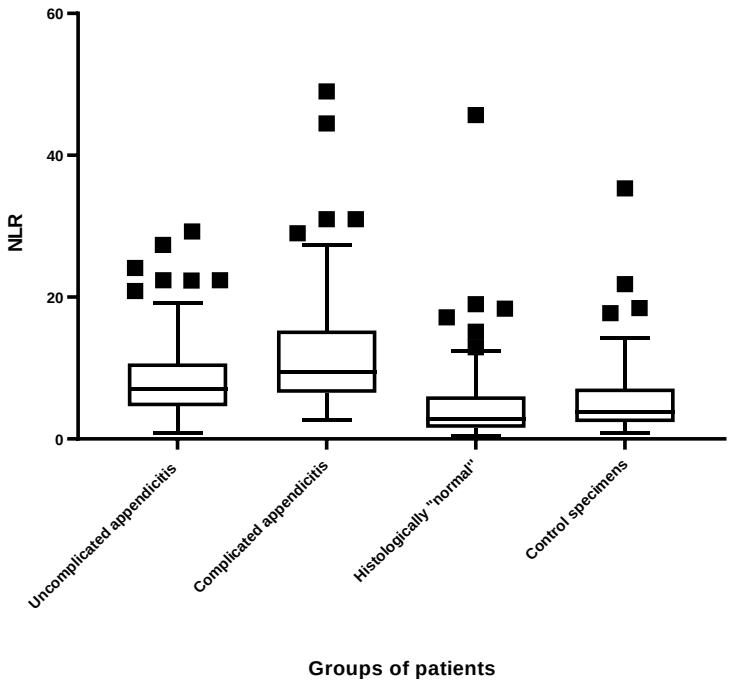
Graph 4.11: Tukey's boxplots displaying data of the lymphocyte count between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 1.6, 25th percentile (Q1) 1.2, 75th percentile (Q3) 2.0, interquartile (IQ) range 0.8; Group II-complicated appendicitis specimens, median 1.2, 25th percentile (Q1) 0.9, 75th percentile (Q3) 1.8, interquartile (IQ) range 0.9; Group III-histologically "normal" specimens, median 2.1, 25th percentile (Q1) 1.3, 75th percentile (Q3) 2.8, interquartile (IQ) range 1.5; Group IV-control specimens, median 1.5, 25th percentile (Q1) 1.1, 75th percentile (Q3) 1.9, interquartile (IQ) range 0.8. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.4 Neutrophil to lymphocyte ratio

The neutrophil to lymphocyte (NLR) ratio was assessed on 435 patients as data was not available on 13 patients. Both groups of patients with acute uncomplicated (median 7.1, IQ range 4.6-10.6, p<0.001) and complicated (median 9.5, IQ range 6.5-15.2, p<0.001) appendicitis demonstrated significantly increased neutrophil to lymphocytes ratio compared with the control group (median 3.7, IQ range 2.3-7.1). Patients with histologically "normal" appendices (median 2.8, IQ range 1.6-6.0) demonstrated a significantly reduced neutrophil to lymphocyte ratio compared with the control group (median 3.7, IQ range 2.3-7.1, p=0.02). Moreover, patients with histologically "normal" (median 2.7, IQ range 1.6-6.0) appendicitis demonstrated significantly reduced neutrophil to lymphocyte ratio compared with group I (median 7.1, IQ range 4.6-10.6, p<0.001) and group II (median 9.5, IQ range 6.5-15.2, p<0.001). Patients with acute uncomplicated (median 7.1, IQ range 4.6-10.6, p<0.05) appendicitis demonstrated a reduced neutrophil to lymphocyte count compared with patients with complicated (median 9.5, IQ range 6.5-15.2, p<0.001) appendicitis (Table 4.12, graph 4.12).

Table 4.12: Summary of the analysis of the neutrophil to lymphocyte ratio between the 4 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	119	7.1	4.6	10.6	6.0	<0.001
II	118	9.5	6.5	15.2	8.7	
III	103	2.8	1.6	6.0	4.4	
IV	95	3.7	2.3	7.1	4.7	



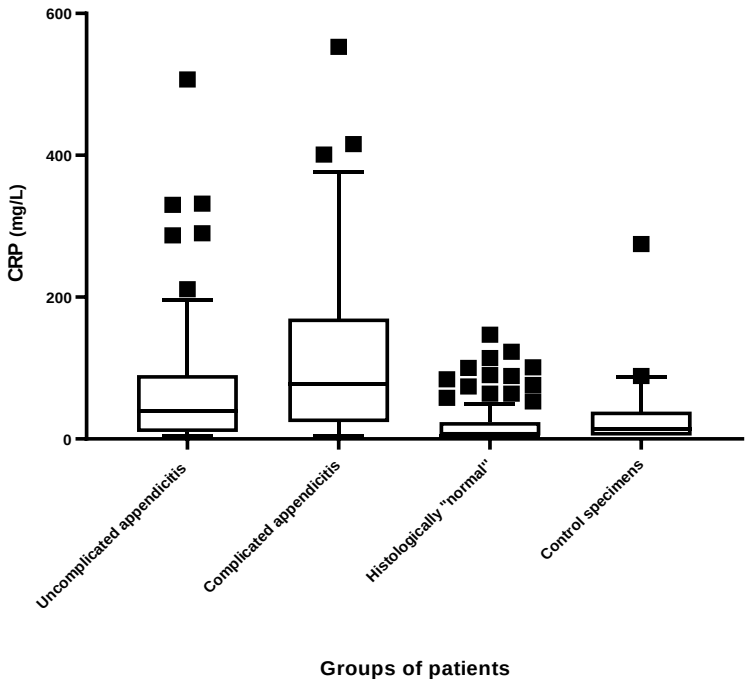
Graph 4.12: Tukey's boxplots displaying data of the neutrophil to lymphocyte ratio (NLR) between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 7.1, 25th percentile (Q1) 4.6, 75th percentile (Q3) 10.6, interquartile (IQ) range 6.0; Group II-complicated appendicitis specimens, median 9.5, 25th percentile (Q1) 6.5, 75th percentile (Q3) 15.2, interquartile (IQ) range 8.7; Group III-histologically "normal" specimens, median 2.8, 25th percentile (Q1) 1.6, 75th percentile (Q3) 6.0, interquartile (IQ) range 4.4; Group IV-control specimens, median 3.7, 25th percentile (Q1) 2.3, 75th percentile (Q3) 7.1, interquartile (IQ) range 4.7. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.5 C-reactive protein concentrations

The C-reactive protein (CRP) concentrations were assessed on 283 patients as data was not available on 165 patients. Patients with complicated (median 77.0, IQ range 24.0-170.0) appendicitis demonstrated significantly increased CRP concentrations compared with the control group (median 18.0, IQ range 5.0-71.0, p=0.001). Patients with acute uncomplicated (median 39.0, IQ range 10.0-90.0) appendicitis did not demonstrate a difference in the CRP concentrations compared with the control (median 18.0, IQ range 5.0-71.0, p=0.10) group. Patients with histologically "normal" appendices (median 5.0, IQ range 5.0-23.7) demonstrated significantly reduced CRP concentrations compared with the control group (median 18.0, IQ range 5.0-71.0, p=0.004). Moreover, both group I (median 39.0, IQ range 10.0-90.0, p<0.001) and group II (median 77.0, IQ range 24.0-170.0, p<0.001) showed significantly increased CRP concentrations compared with group III (median 5.0, IQ range 5.0-23.7). Patients with complicated (median 77.0, IQ range 24.0-170.0) appendicitis demonstrated significantly higher CRP concentrations compared with patients with acute uncomplicated (median 39.0, IQ range 10.0-90.0, p=0.001) appendicitis (Table 4.13, graph 4.13).

Table 4.13: Summary of the analysis of the CRP concentrations between the 4 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices, Group IV-patients of the control group. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 4 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	P value
I	93	39.0	10.0	90.0	80.0	<0.001
II	79	77.0	24.0	170.0	146.0	
III	80	5.0	5.0	23.7	18.7	
IV	31	18.0	5.0	71.0	66.0	



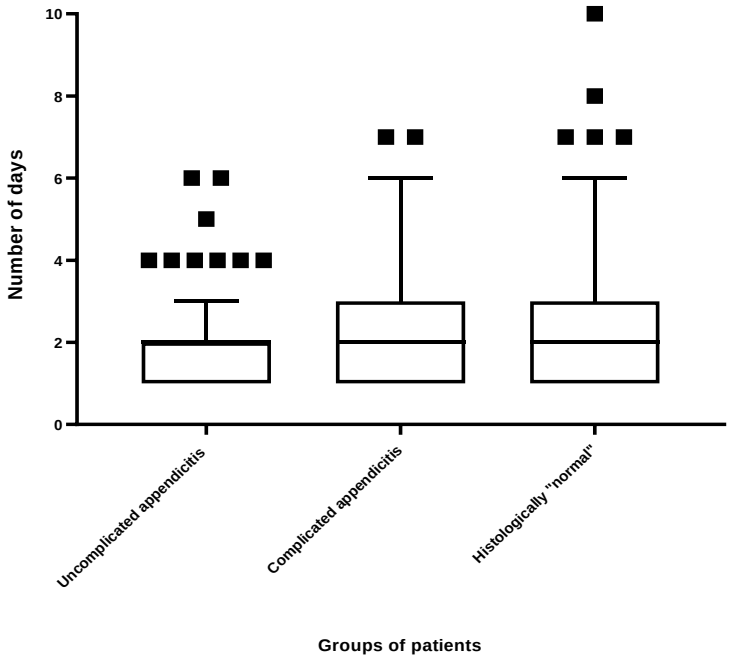
Graph 4.13: Tukey's boxplots displaying data of the CRP concentrations between the 4 groups of specimens. Group I-uncomplicated appendicitis specimens, median 39.0, 25th percentile (Q1) 10.0, 75th percentile (Q3) 90.0.0, interquartile (IQ) range 80.0; Group II-complicated appendicitis specimens, median 77.0, 25th percentile (Q1) 24.0, 75th percentile (Q3) 170.0, interquartile (IQ) range 146.0; Group III-histologically "normal" specimens, median 5.0, 25th percentile (Q1) 5.0, 75th percentile (Q3) 23.7, interquartile (IQ) range 18.7; Group IV-control specimens, median 18.0, 25th percentile (Q1) 5.0, 75th percentile (Q3) 71.0, interquartile (IQ) range 66.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.6 Duration of symptoms

The duration of symptoms suggesting acute appendicitis was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" samples by reviewing the clinical notes on the day of admission to hospital. The duration of symptoms was assessed on 313 patients as data was not available on 30 patients. Patients with histologically "normal" (median 2.0, IQ range 1.0-3.0) appendices did not demonstrate significant difference in the duration of symptoms suggesting appendicitis compared with the uncomplicated acute (median 2.0, IQ range 1.0-2.0, p=0.11) appendicitis or complicated appendicitis (median 2.0, IQ range 1.0-3.0, p=0.18) samples. Moreover, patients with acute (median 2.0, IQ range 1.0-2.0) appendicitis did not demonstrate significantly different duration of symptoms compared with patients with complicated (median 2.0, IQ range 1.0-3.0, p=0.87) appendicitis (Table 4.14, graph 4.14).

Table 4.14: Summary of the analysis of the duration of symptoms between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	111	2.0	1.0	2.0	1.0	0.62
II	108	2.0	1.0	3.0	2.0	
III	93	2.0	1.0	3.0	2.0	



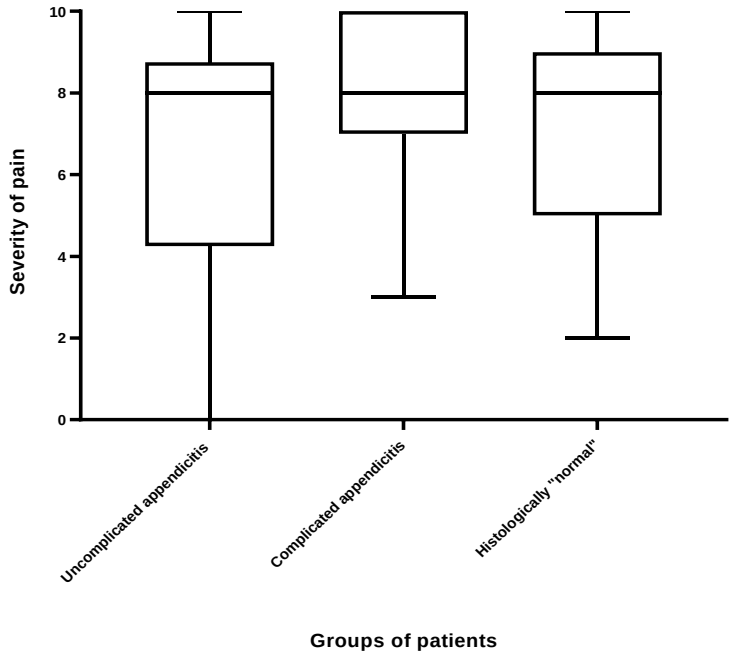
Graph 4.14: Tukey's boxplots displaying data of the duration of symptoms between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, median 2.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 2.0, interquartile (IQ) range 1.0; Group II-patients with complicated appendicitis, median 2.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 3.0, interquartile (IQ) range 2.0; Group III-patients with histologically "normal" appendices, median 2.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 3.0, interquartile (IQ) range 2.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.7 Severity of pain

The severity of pain was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" samples by reviewing the clinical notes on the day of admission to hospital. The patient was asked to rate the pain on a scale of 0 to 10 with 0 being no pain and 10 being the worst pain ever experienced. The severity of pain was assessed on 301 patients as data was not available on 41 patients. Patients with histologically "normal" (median 8.0, IQ range 5.0-9.0) appendices did not demonstrate significant difference in the severity of pain compared with the acute (median 8.0, IQ range 4.2-8.7, p=0.68) or complicated (median 8.0, IQ range 7.0-10.0, p=0.07) appendicitis patients. Moreover, patients with acute (median 8.0, IQ range 4.2-8.7) appendicitis demonstrated significantly reduced severity of pain compared with patients with complicated (median 8.0, IQ range 7.0-10.0, p=0.01) appendicitis (Table 4.15, graph 4.15).

Table 4.15: Summary of the analysis of the severity of pain between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	108	8.0	4.2	8.7	4.5	0.04
II	105	8.0	7.0	10.0	3.0	
III	88	8.0	5.0	9.0	4.0	



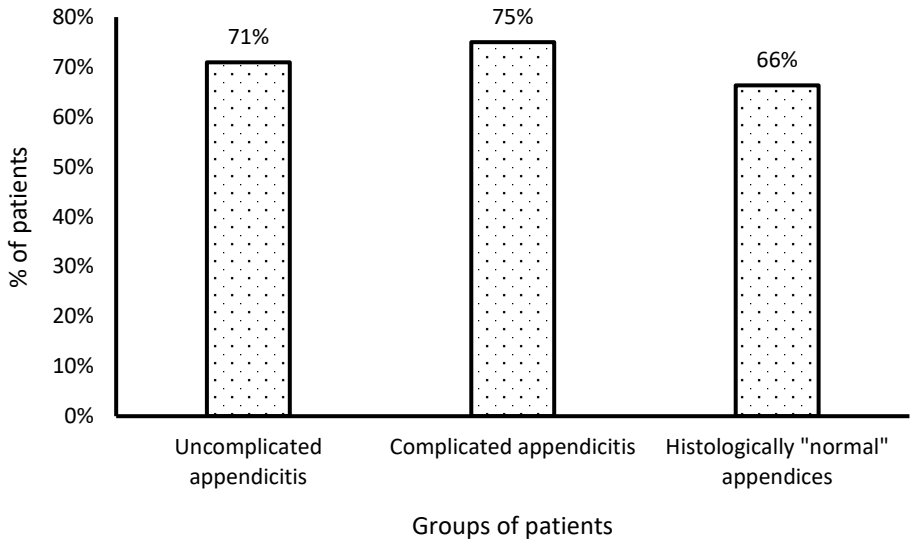
Graph 4.15: Tukey's boxplots displaying data of the severity of pain between the 3 groups of patients. Group I-patients uncomplicated appendicitis, median 8.0, 25th percentile (Q1) 4.2, 75th percentile (Q3) 8.7, interquartile (IQ) range 4.5; Group II-patients with complicated appendicitis, median 8.0, 25th percentile (Q1) 7.0, 75th percentile (Q3) 10.0, interquartile (IQ) range 3.0; Group III-patients with histologically "normal" appendices, median 8.0, 25th percentile (Q1) 5.0, 75th percentile (Q3) 9.0, interquartile (IQ) range 4.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.8 Nausea and/or vomiting

The symptoms of nausea and/or vomiting were assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" samples by reviewing the clinical notes on the day of admission to hospital. The symptoms of nausea and/or vomiting were assessed on 313 patients as data was not available on 29 patients. In the acute appendicitis group, 78 (71%) patients complained of nausea and/or vomiting. In the complicated appendicitis group, 81 (75%) patients complained of nausea and/or vomiting, whereas, in the group of patients with histologically normal appendices 63 (66%) patients complained of nausea and/or vomiting (Table 4.16, graph 4.16).

Table 4.16: Summary of the presence of nausea/vomiting between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. Statistically significant difference between the 3 groups was calculated using the Pearson's Chi-Square test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Presence of nausea or vomiting				
Groups	**Number of patients assessed**	**Number of patients with nausea or vomiting**	**%**	**Pearson's Chi-Square**
I	110	78	71%	p=0.397
II	108	81	75%	
III	95	63	66%	
Total	313	222		



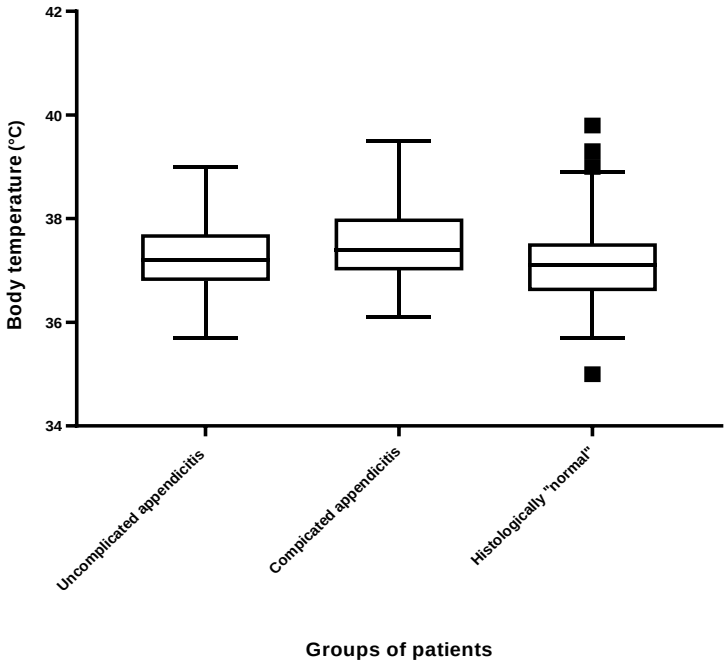
Graph 4.16: The presence of nausea or vomiting between the 3 groups of patients.

4.3.9 Body temperature on admission

The body temperature on admission was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" by reviewing the first recorded temperature on admission to the Accident and Emergency department or the letter from the General Practitioner who referred the patient to the Surgical Admission Unit. Therefore, the body temperature was not affected by antipyretic medication such as paracetamol. The body temperature on admission was assessed on 312 patients as data was not available on 30 patients. Patients with histologically "normal" (median 37.1, IQ range 36.6-37.5) appendices did not demonstrate significant difference in the first recorded body temperature compared with the uncomplicated acute (median 37.2, IQ range 36.8-37.7, p=0.16) appendicitis patients. Patients with complicated (median 37.4, IQ range 37.0-38.0) appendicitis demonstrated significantly increased body temperature on admission compared with patients with histologically "normal" appendices (median 37.1, IQ range 36.6-37.5, p=0.002) as well as with the acute (median 37.2, IQ range 36.8-37.7, p=0.047) appendicitis patients. However, the latter was weekly significant (Table 4.17, graph 4.17).

Table 4.17: Summary of the analysis of body temperature on admission between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	110	37.2	36.8	37.7	0.9	0.005
II	108	37.4	37.0	38.0	1.0	
III	94	37.1	36.6	37.5	0.9	



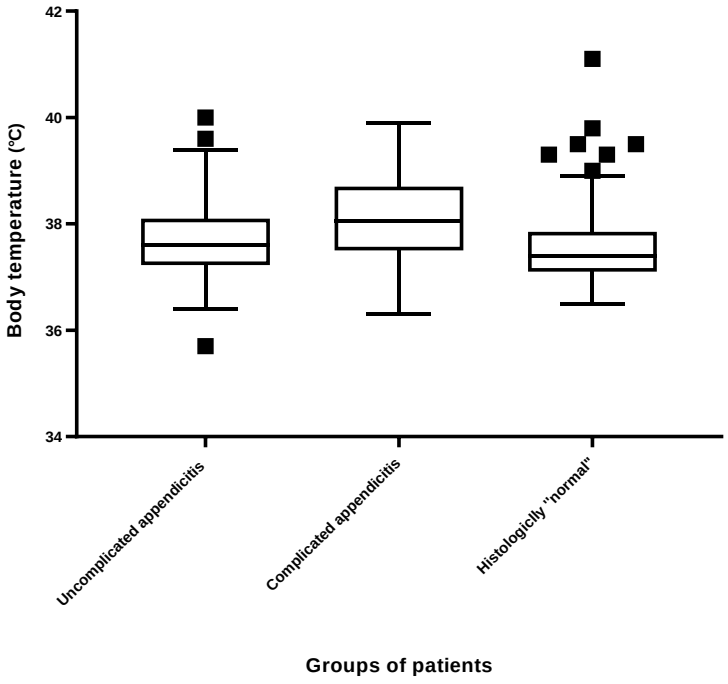
Graph 4.17: Tukey's boxplots displaying data of body temperature on admission between the 3 groups of patients. Group I-patients uncomplicated appendicitis, median 37.2, 25th percentile (Q1) 36.8, 75th percentile (Q3) 37.7, interquartile (IQ) range 0.9; Group II-patients with complicated appendicitis, median 37.4, 25th percentile (Q1) 37.0, 75th percentile (Q3) 38.0, interquartile (IQ) range 1.0; Group III-patients with histologically "normal" appendices, median 37.1, 25th percentile (Q1) 36.6, 75th percentile (Q3) 37.5, interquartile (IQ) range 0.9. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.10 Highest body temperature pre-operatively

The highest body temperature during admission was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" by recording the highest temperature in the pre-operative period. The highest body temperature was assessed on 311 patients as data was not available on 31 patients. The highest recorded temperature pre-operatively in patients with histologically "normal" (median 37.4, IQ range 37.1-37.8) appendices was significantly lower compared with acute (median 37.6, IQ range 37.2-38.1, p=0.02) appendicitis as well as with complicated (median 38.0, IQ range 37.5-38.7, p<0.001) appendicitis patients. The highest recorded temperature pre-operatively in patients with complicated (median 38.0, IQ range 37.5-38.7) appendicitis was significantly higher compared with patients with acute (median 37.6, IQ range 37.2-38.1, p<0.001) appendicitis (Table 4.18, graph 4.18).

Table 4.18: Summary of the analysis on the highest body temperature pre-operatively between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	119	37.6	37.2	38.1	0.9	<0.001
II	108	38.0	37.5	38.7	1.2	
III	94	37.4	37.1	37.8	0.7	



Graph 4.18 Tukey's boxplots displaying data on the highest body temperature pre-operatively between the 3 groups of patients. Group I-patients uncomplicated appendicitis, median 37.6, 25th percentile (Q1) 37.2, 75th percentile (Q3) 38.1, interquartile (IQ) range 0.9; Group II-patients with complicated appendicitis, median 38.0, 25th percentile (Q1) 37.5, 75th percentile (Q3) 38.7, interquartile (IQ) range 1.2; Group III-patients with histologically "normal" appendices, median 37.4, 25th percentile (Q1) 37.1, 75th percentile (Q3) 37.8, interquartile (IQ) range 0.7. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

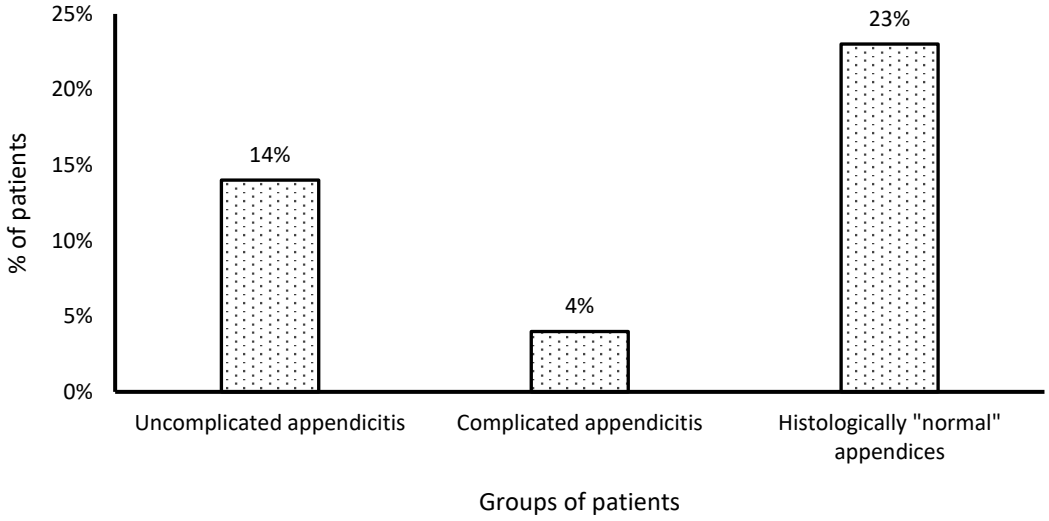
4.3.11 Previous episodes of right iliac fossa pain

The presence of previous episodes of right iliac fossa pain was assessed in acute appendicitis, complicated appendicitis and histologically "normal" appendices by reviewing the clinical notes on the day of admission to hospital. Therefore, the presence of previous episodes of right iliac fossa pain was assessed on 317 patients as data was not available on 25 patients. In the acute appendicitis group, 16 (14%) patients were found to have previous episodes of right iliac fossa pain. In the complicated appendicitis group, 4 (4%) patients reported to have previous episodes of right iliac fossa pain. Moreover, in the histologically "normal" samples 22 (23%) patients were found to have previous episodes of right iliac fossa pain. The observed differences between the three groups were statistically significant (Table 4.19, graph 4.19).

The expression of the studied inflammatory markers (TNF-α, IL-6, IL-2R and serotonin) as well as the levels of various clinical parameters (WCC, NLR, CRP, severity of pain, duration of symptoms, body temperature on admission and highest body temperature) were assessed between patients with and without previous episodes of right iliac fossa pain in the groups of acute uncomplicated appendicitis, acute complicated appendicitis and in patients with histologically "normal" appendices. The observed differences between patients with and without previous episodes of right iliac fossa pain were not statistically significant in any of the studied parameters. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error (Tables 4.20-4.22).

Table 4.19: Summary of the presence of previous episodes of right iliac fossa pain between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. Statistically significant difference between the 3 groups was calculated using the Pearson's Chi-Square test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Previous episodes of right iliac fossa pain				
Groups	**Number of patients assessed**	**Number of patients with previous episodes of RIF pain**	**%**	**Pearson's Chi-Square**
I	112	16	14%	p<0.001
II	109	4	4%	
III	96	22	23%	
Total	317	69		



Graph 4.19: The presence previous episodes of right iliac fossa pain between the 3 groups of patients.

Table 4.20: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with acute uncomplicated appendicitis due to previous episodes of right iliac fossa pain. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the CRP concentrations (*) was not statistically significant.

Previous episodes of RIF pain in uncomplicated appendicitis and other variables				
Variable		**With previous RIF pain median (25^{th}-75^{th})**	**Without previous RIF pain median (25^{th}-75^{th})**	**p value**
TNF-α		6.0 (2.6-10.4)	5.9 (3.4-9.6)	0.86
IL-6	**Epithelial**	19.0 (1.5-63.7)	8.0 (1.1-52.0)	0.63
	Inflammatory	4.8 (1.9-16.6)	8.3 (3.4-17.7)	0.37
IL-2R	**Mucosa**	44.2 (25.1-84.2)	45.7 (36.0-68.2)	0.60
	Submucosa	61.4 47.5-81.1)	67.8 (50.5-88.5)	0.63
Serotonin	**ECCs**	6.5 (0-45.3)	3.0 (0-27.0)	0.46
	SNCs	0 (0-4.6)	0 (0-2.0)	0.49
WCC		12.5 (11.0-14.9)	13.7 (11.5-16.2)	0.26
NLR		7.7 (3.2-10.6)	7.1 (4.6-10.7)	0.66
CRP		14.5 (5.0-63.0)	40.0 (19.5-95.0)	**0.03***
Severity of pain		8.0 (6.0-10.0)	8.0 (4.0-8.0)	0.25
Duration of symptoms		2.0 (1.0-3.7)	2.0 (1.0-2.0)	0.08
Temperature on admission		37.4 (36.8-37.8)	37.1 (36.7-37.6)	0.38
Highest temperature pre-operatively		37.8 (37.2-38.8)	37.6 (37.2-38.0)	0.24

Table 4.21: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with acute complicated appendicitis due to previous episodes of right iliac fossa pain. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed differences in the severity of pain and duration of symptoms (*) were not statistically significant. In this group of patients several values of CRP concentrations (**) were not available and as result, statistical analysis could not be performed.

Previous episodes of RIF pain in complicated appendicitis and other variables				
Variable		**With previous RIF pain median (25^{th}-75^{th})**	**Without previous RIF pain median (25^{th}-75^{th})**	**p value**
TNF-α		4.2 (4.1- -)	7.0 (3.5-11.8)	0.57
IL-6	**Epithelial**	50.0 (45.0- -)	51.0 (8.5-103.0)	0.46
	Inflammatory	19.3 (18.0- -)	24.7 (10.0-49.5)	0.70
IL-2R	**Mucosa**	46.2 (44.4- -)	36.5 (25.3-60.4)	0.49
	Submucosa	58.7 (32.1-78.5)	48.4 (30.3-68.5)	0.66
Serotonin	**ECCs**	25.5 (0- -)	0 (0-9.3)	0.22
	SNCs	1.0 (0- -)	0 (0-0)	0.18
WCC		15.2 (12.7-20.8)	15.2 (12.3-18.5)	0.74
NLR		6.3 (37-12.9)	10.1 (6.5-15.5)	0.22
CRP**		N/A	N/A	N/A
Severity of pain		4.2 (4.0-6.5)	7.0 (4.0-8.0)	0.14
Duration of symptoms		1.0 (1.0-1.0)	1.0 (2.0-3.0)	**0.03***
Temperature on admission		36.7 (36.2-37.1)	37.4 (37.0-38.0)	**0.02***
Highest temperature pre-operatively		37.6 (37.2-38.5)	38.1 (37.5-38.7)	0.41

Table 4.22: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with histologically "normal" appendices due to previous episodes of right iliac fossa pain. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the CRP concentrations (*) was not statistically significant.

Previous episodes of RIF pain in histologically "normal" appendices and other variables				
Variable		**With previous RIF pain median (25^{th}-75^{th})**	**Without previous RIF pain median (25^{th}-75^{th})**	**p value**
TNF-α		11.4 (7.0-16.1)	9.6 (5.8-15.2)	0.29
IL-6	**Epithelial**	69.0 (7.6-172.5)	74.5 (24.0-130.5)	0.88
	Inflammatory	27.0 (6.1-52.7)	20.5 (11.3-41.2)	0.93
IL-2R	**Mucosa**	27.2 (22.3-43.2)	28.3 (19.5-43.6)	0.85
	Submucosa	16.8 (7.6-31.8)	21.6 (10.4-36.9)	0.40
Serotonin	**ECCs**	33.3 (14.5-113.5)	43.4 (16.3-95.5)	0.96
	SNCs	2.0 (0-6.8)	1.0 (0-4.0)	0.36
WCC		9.5 (6.3-10.5)	10.2 (7.4-13.3)	0.97
NLR		1.9 (1.2-4.7)	3.3 (1.7-6.3)	0.73
CRP		5.0 (5.0-8.7)	7.0 (5.0-48.5)	**0.03***
Severity of pain		8.0 (5.5-10.0)	8.0 (5.0-9.0)	0.51
Duration of symptoms		2.0 (1.0-3.5)	2.0 (1.0-3.0)	0.62
Temperature on admission		37.1 (36.6-37.4)	37.1 (36.6-37.6)	0.84
Highest temperature pre-operatively		37.2 (37.1-37.7)	37.4 (37.1-37.9)	0.37

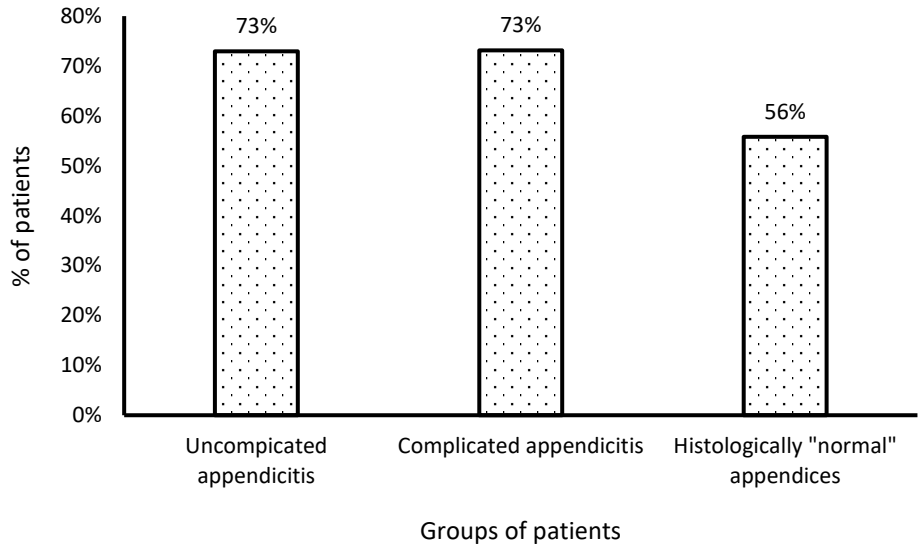
4.3.12 Localised peritonism

The presence of localised peritonism in the right iliac fossa was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" samples by reviewing the clinical notes on the day of admission to hospital. Documentation of presence of guarding and/or rebound tenderness by surgical registrars or consultants was considered as localised peritonism. The sign of peritonism was assessed on 314 patients as data was not available on 28 patients. In the acute appendicitis group, 81 (73%) patients were found to have localised peritonism. In the complicated appendicitis group, 79 (73%) patients were found to have peritonism in the right iliac fossa. Moreover, in the group of patients with histologically normal appendices, 53 (56%) patients were found to have localised peritonism on examination. The observed differences between the three groups were statistically significant (Table 4.23, graph 4.20).

The expression of the studied inflammatory markers (TNF-α, IL-6, IL-2R and serotonin) as well as the levels of various clinical parameters (WCC, NLR, CRP, severity of pain, duration of symptoms, body temperature on admission and highest body temperature) were assessed between patients with and without localised peritonism in the groups of acute uncomplicated appendicitis, acute complicated appendicitis and in patients with histologically "normal" appendices. The observed differences between patients with and without the localised peritonism were not statistically different in any of the studied parameters. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error (Tables 4.24-4.26).

Table 4.23: Summary of the presence of localised peritonism in the right iliac fossa between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. Statistically significant difference between the 3 groups was calculated using the Pearson's Chi-Square test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Presence of localised peritonism				
Groups	**Number of patients assessed**	**Number of patients with localised peritonism**	**%**	**Pearson's Chi-Square**
I	111	81	73%	p=0.01
II	108	79	73%	
III	95	53	56%	
Total	314	213		



Graph 4.20: The presence of localised peritonism in the right iliac fossa between the 3 groups of patients.

Table 4.24: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with uncomplicated appendicitis due to the presence of localised peritonism in the right iliac fossa. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the duration of symptoms (*) was not statistically significant.

Presence of localised peritonism in uncomplicated appendicitis and other variables				
Variable		**With localised peritonism median (25^{th}-75^{th})**	**Without localised peritonism median (25^{th}-75^{th})**	**p value**
TNF-α		6.0 (3.1-9.9)	5.9 (3.4-8.8)	0.78
IL-6	**Epithelial**	9.0 (1.0-71.5)	7.0 (3.0-51.0)	0.82
	Inflammatory	6.8 (3.0-16.7)	11.6 (1.8-19.0)	0.74
IL-2R	**Mucosa**	47.4 (38.3-67.4)	40.8 (30.8-70.6)	0.26
	Submucosa	67.8 (50.2-89.5)	65.7 (51.4-79.9)	0.60
Serotonin	**ECCs**	4.0 (0-36.0)	2.0 (0-9.0)	0.15
	SNCs	0 (0-2.5)	0 (0-2.0)	0.54
WCC		13.6 (11.4-16.3)	13.7 (12.2-15.2)	0.99
NLR		7.4 (4.5-10.7)	6.1 (4.7-9.3)	0.29
CRP		37.5 (16.0-88.5)	33.0 (5.0-72.2)	0.46
Severity of pain		8.0 (5.0-9.0)	7.0 (4.0-8.0)	0.20
Duration of symptoms		2.0 (1.0-3.0)	1.0 (1.0-2.0)	**0.02***
Temperature on admission		37.3 (36.8-37.7)	37.0 (36.7-37.6)	0.13
Highest temperature pre-operatively		37.6 (37.3-38.2)	37.5 (37.2-37.9)	0.25

Table 4.25: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with complicated appendicitis due to the presence of localised peritonism in the right iliac fossa. The observed differences were not statistically significant.

Presence of localised peritonism in complicated appendicitis and other variables				
Variable		**With localised peritonism median (25^{th}-75^{th})**	**Without localised peritonism median (25^{th}-75^{th})**	**p value**
TNF-α		7.5 (4.2-11.3)	5.9 (3.4-13.6)	0.99
IL-6	**Epithelial**	50.0 (8.7-111.2)	61.0 (8.6-92.0)	0.90
	Inflammatory	24.0 (10.9-50.6)	22.4 (8.6-51.6)	0.84
IL-2R	**Mucosa**	38.4 (25.0-60.7)	35.0 (29.9-44.5)	0.52
	Submucosa	46.6 (29.8-67.0)	55.6 (34.0-77.9)	0.23
Serotonin	**ECCs**	0 (0-12.0)	0 (0-1.5)	0.23
	SNCs	0 (0-0)	0 (0-1.0)	0.74
WCC		15.1 (11.9-18.0)	15.4 (12.6-19.1)	0.39
NLR		10.6 (6.4-14.7)	8.5 (6.2-17.9)	0.54
CRP		85 (21.0-195.0)	73.0 (47.0-162.0)	0.98
Severity of pain		8.0 (7.0-10.0)	8.0 (7.0-9.0)	0.42
Duration of symptoms		2.0 (1.0-3.0)	2.0 (1.0-2.0)	0.14
Temperature on admission		37.4 (37.0-38.0)	37.3 (37.0-37.7)	0.61
Highest temperature pre-operatively		38.1 (37.5-38.7)	37.8 (37.4-38.6)	0.30

Table 4.26: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with histologically "normal" appendices due to the presence of localised peritonism in the right iliac fossa. The observed differences were not statistically significant.

Presence of localised peritonism in histologically "normal" appendices and other variables				
Variable		**With localised peritonism median (25^{th}-75^{th})**	**Without localised peritonism median (25^{th}-75^{th})**	**p value**
TNF-α		9.7 (6.8-15.3)	9.7 (5.5-14.6)	0.56
IL-6	**Epithelial**	81.0 (19.7-131.0)	70.0 (20.2-142.5)	0.97
	Inflammatory	20.0 (10.2-40.1)	27.0 (11.0-44.3)	0.50
IL-2R	**Mucosa**	30.0 (22.8-45.5)	24.3 (1831-39.9)	0.98
	Submucosa	22.8 (10.2-37.7)	17.0 (7.6-34.5)	0.26
Serotonin	**ECCs**	33.6 (13.1-86.9)	45.5 (1937-115.0)	0.47
	SNCs	1.0 (0-5.4)	1.0 (0-5.2)	0.87
WCC		10.0 (7.5-12.6)	10.1 (7.2-13.1)	0.82
NLR		2.9 (1.9-5.8)	2.9 (1.4-8.6)	0.90
CRP		5.0 (5.0-25.0)	6.0 (5.0-43.7)	0.64
Severity of pain		8.0 (6.0-10.0)	8.0 (4.0-8.5)	0.20
Duration of symptoms		2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.50
Temperature on admission		37.1 (36.6-37.6)	37.1 (36.5-37.5)	0.85
Highest temperature pre-operatively		37.3 (37.1-37.9)	37.5 (37.1-37.8)	0.60

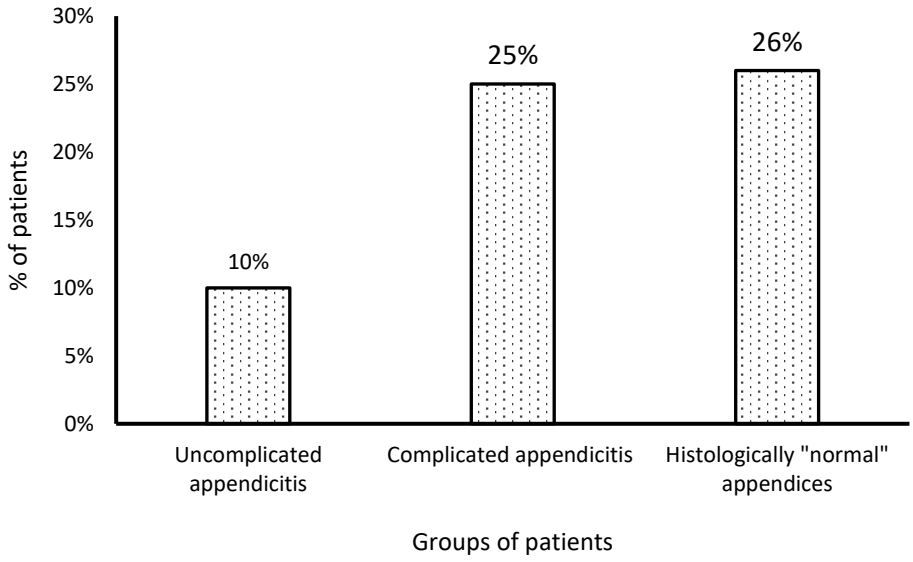
4.3.13 Presence of faecolith

The presence of faecolith was assessed in the acute uncomplicated appendicitis, complicated appendicitis and histologically "normal" appendices by reviewing the imaging results (USS or CT), the operative record and the histopathology report. Therefore, the presence of faecolith was assessed on 317 patients as data was not available on 25 patients. In the acute uncomplicated appendicitis group, 12 (10%) patients were found to have a faecolith within the lumen of the appendix. In the complicated appendicitis group, 30 (25%) patients were found to have a faecolith with the lumen of the appendix. Moreover, 27 (26%) patients were found to have a faecolith in the histologically "normal" samples. The observed differences between the three groups were statistically significant (Table 4.27, graph 4.21).

The expression of the studied inflammatory markers (TNF-α, IL-6, IL-2R and serotonin) as well as the levels of various clinical parameters (WCC, NLR, CRP, severity of pain, duration of symptoms, body temperature on admission and highest body temperature) were assessed between patients with and without faecolith in the groups of acute uncomplicated appendicitis, acute complicated appendicitis and in patients with histologically "normal" appendices. The observed differences between patients with and without faecolith were not statistically different in any of the studied parameters. Due to multiple statistical testing, a new Bonferroni-adjusted p value (0.0035) was applied to control for Type I error (Tables 4.28-4.30).

Table 4.27: Summary of the presence of faecolith between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. Statistically significant difference between the 3 groups was calculated using the Pearson's Chi-Square test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Presence of faecolith				
Groups	**Number of patients assessed**	**Number of patients with faecolith**	**%**	**Pearson's Chi-Square**
I	112	12	10%	p=0.003
II	109	30	25%	
III	96	27	26%	
Total	317	69		



Graph 4.21: The presence of faecolith within the lumen of the appendix between the three groups of patients.

Table 4.28: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with acute uncomplicated appendicitis due to the presence of faecolith. The observed differences were not statistically significant.

Presence of faecolith in uncomplicated appendicitis and other variables				
Variable		**With faecolith median (25^{th}-75^{th})**	**Without faecolith median (25^{th}-75^{th})**	**p value**
TNF-α		6.8 (3.6-12.2)	5.9 (3.3-9.6)	0.46
IL-6	**Epithelial**	31.5 (731-123.0)	8.0 (1.0-52.0)	0.13
	Inflammatory	8.0 (1.8-26.9)	7.3 (3.0-17.0)	0.85
IL-2R	**Mucosa**	54.5 (32.2-86.5)	45.8 (35.2-68.2)	0.68
	Submucosa	68.4 (41.1-89.5)	67.9 (52.6-87.9)	0.78
Serotonin	**ECCs**	1.0 (0-29.5)	3.0 (0-30.0)	0.57
	SNCs	0 (0-1.5)	0 (0-2.2)	0.36
WCC		13.6 (11.9-17.7)	13.6 (11.2-16.0)	0.72
NLR		7.7 (4.8-12.4)	7.1 (4.6-10.6)	0.66
CRP		58.0 (13.5-120.2)	39.0 (11.5-87.0)	0.54
Severity of pain		7.5 (4.0-10.0)	8.0 (5.0-8.0)	0.91
Duration of symptoms		1.0 (1.0-3.5)	2.0 (1.0-2.0)	0.47
Temperature on admission		37.3 (36.7-37.6)	37.2 (36.8-37.7)	0.97
Highest temperature pre-operatively		37.7 (37.4-37.9)	37.6(37.2-38.1)	0.34

Table 4.29: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with acute complicated appendicitis due to the presence of faecolith. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the CRP concentrations (*) was not statistically significant.

Presence of faecolith in complicated appendicitis and other variables				
Variable		**With faecolith median (25^{th}-75^{th})**	**Without faecolith median (25^{th}-75^{th})**	**p value**
TNF-α		7.0 (3.2-11.3)	6.6 (3.7-12.5)	0.39
IL-6	**Epithelial**	65.0 (20.0-135.0)	27.5 (5.7-94.2)	0.64
	Inflammatory	31.1 (18.0-61.1)	20.0 (9.5-39.6)	0.91
IL-2R	**Mucosa**	35.6 (18.4-60.3)	38.4 (26.9-58.4)	0.51
	Submucosa	52.4 (30.5-70.2)	47.0 (31.1-69.5)	0.52
Serotonin	**ECCs**	0 (0-8.7)	0 (0-8.5)	0.74
	SNCs	0 (0-0)	0 (0-0)	0.63
WCC		14.9 (9.9-18.5)	15.4 (13.1-1834)	0.20
NLR		9.7 (5.2-16.4)	9.2 (6.4-14.7)	0.79
CRP		147.0 (72.0-229)	65.5 (20.0-161.5)	**0.02***
Severity of pain		8.0 (7.2-9.0)	8.0 (7.0-10.0)	0.90
Duration of symptoms		2.0 (1.0-3.0)	2.0 (1.0-2.0)	0.19
Temperature on admission		37.4 (37.0-38.3)	37.4 (37.0-37.9)	0.43
Highest temperature pre-operatively		38.5 (37.6-38.7)	38.0 (37.5-38.6)	0.21

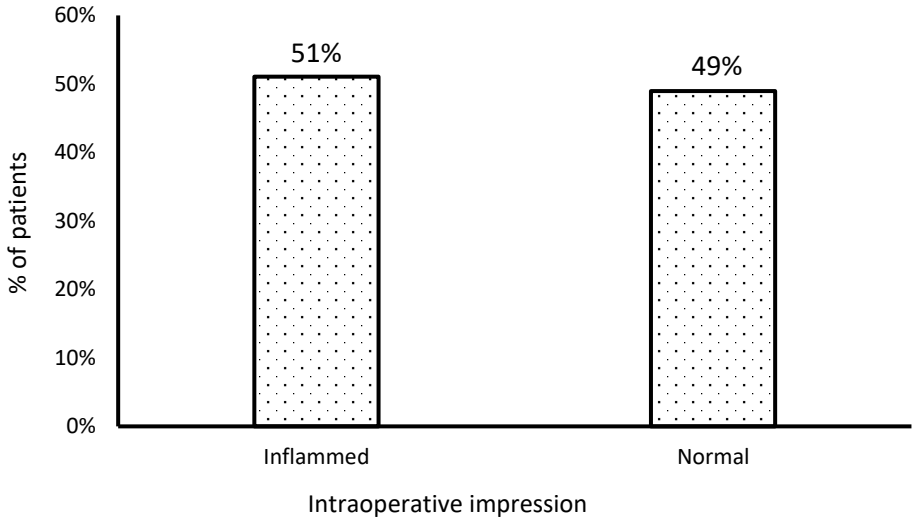
Table 4.30: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with histologically "normal" appendices due to the presence of faecolith. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the epithelial expression of IL-6 (*) was not statistically significant.

Presence of faecolith in histologically "normal" appendices and other variables				
Variable		**With faecolith median (25^{th}-75^{th})**	**Without faecolith median (25^{th}-75^{th})**	**p value**
TNF-α		11.2 (6.7-16.1)	9.7 (6.9-14.4)	0.50
IL-6	**Epithelial**	29.5 (11.0-87.0)	82.5 (30.0-150.0)	**0.04***
	Inflammatory	20.0 (5.8-42.0)	21.5 (11.5-43.4)	0.33
IL-2R	**Mucosa**	26.4 (19.6-44.4)	27.2 (20.4-39.9)	0.87
	Submucosa	13.6 (7.0-27.0)	21.6 (12.0-39.6)	0.05
Serotonin	**ECCs**	25.0 (13.2-96.0)	53.5 (19.1-112.3)	0.27
	SNCs	1.0 (0-3.0)	2.0 (0-0.6)	0.13
WCC		9.5 (7.3-11.3)	10.1 (7.0-13.0)	0.89
NLR		3.3 (1.65-7.1)	2.6 (1.5-5.8)	0.40
CRP		5.0 (5.0-36.0)	5.0 (5.0-24.0)	0.98
Severity of pain		8.0 (4.5-10.0)	8.0 (5.0-9.0)	0.72
Duration of symptoms		2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.68
Temperature on admission		37.1 (36.7-37.5)	37.1 (36.5-37.5)	0.76
Highest temperature pre-operatively		37.3 (37.0-37.8)	37.4 (37.1-37.8)	0.42

4.3.14 Intraoperative diagnosis

The intraoperative impression of the operating surgeons, whether the appendix appeared to be inflamed or not, was assessed in the histologically "normal" specimens. Expressions such as injected appendix, slightly engorged appendix, hyper-vascular appendix were classified as inflamed appendix. The intraoperative diagnosis was available on 96 patients as data was not available on 8 patients. In 49 (51%) of 96 patients who subsequently had a histologically "normal" appendix, the operating surgeons described the appendix as inflamed (Graph 4.22).

The expression of the studied inflammatory markers (TNF-α, IL-6, IL-2R and serotonin) as well as the levels of various clinical parameters (WCC, NLR, CRP, severity of pain, duration of symptoms, body temperature on admission and highest body temperature) were assessed between patients whose appendix appeared normal and those whose appendix appeared inflamed in the groups of acute uncomplicated appendicitis, acute complicated appendicitis and in patients with histologically "normal" appendices. The observed differences between patients whose appendix appeared normal and those whose appendix appeared inflamed were not statistically different in any of the studied parameters. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error (Table 4.31).



Graph 4.22: Intraoperative impression of the histologically "normal" appendices.

Table 4.31: Summary of the differences in the expression of the studied inflammatory markers and the levels of clinical parameters in patients with histologically "normal" appendices according to the intraoperative diagnosis. Due to multiple statistical testing, a Bonferroni-adjusted p value (0.0035) was applied to control for Type I error. Therefore, the observed difference in the highest body temperature pre-operatively (*) was not statistically significant.

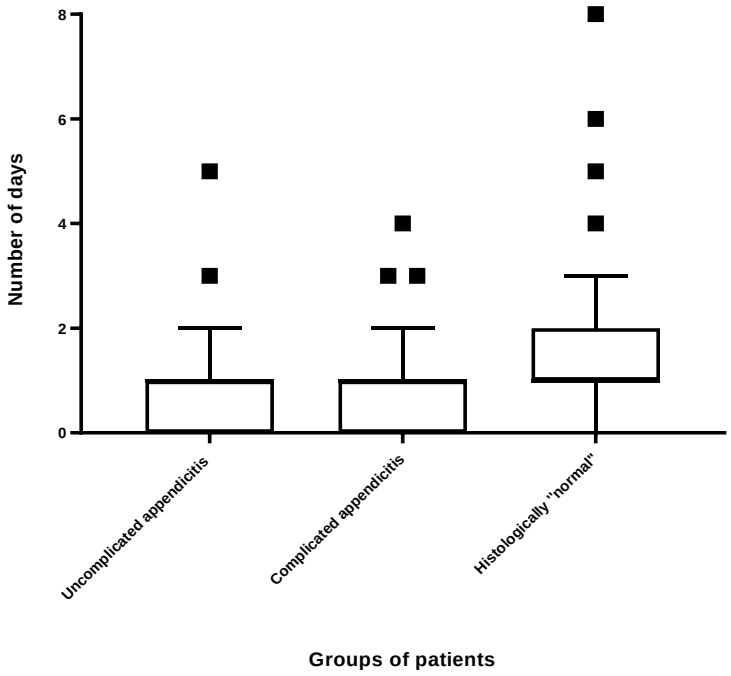
Intraoperative diagnosis in histologically "normal" appendices and other variables				
Variable		**Appeared normal median (25^{th}-75^{th})**	**Appeared inflamed median (25^{th}-75^{th})**	**p value**
TNF-α		8.4 (6.1-12.8)	11 (6.3-16.1)	0.06
IL-6	**Epithelial**	71 (21.0-140.0)	78.0 (18.2-120.0)	0.77
	Inflammatory	21.0 (8.6-43.0)	22.0 (10.7-42.1)	0.94
IL-2R	**Mucosa**	26.2 (1934-39.0)	30.0 (21.4-46.3)	0.20
	Submucosa	21.6 (12.0-30.8)	17.6 (7.4-39.7)	0.80
Serotonin	**ECCs**	61.5 (17.5-116.0)	33.0 (12.1-86.9)	0.97
	SNCs	1.0 (0-6.0)	1.0 (0-4.0)	0.63
WCC		10.1 (6.9-13.5)	10.0 (7.4-12.3)	0.82
NLR		4.0 (1.6-6.9)	2.6 (1.5-5.3)	0.15
CRP		5.0 (5.0-37.0)	5.0 5.0-29.2)	0.76
Severity of pain		8.0 (6.5-10.0)	8.0 (4.0-8.0)	0.12
Duration of symptoms		2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.14
Temperature on admission		36.6 (36.2-37.2)	36.5 (36.2-37.1)	0.36
Highest temperature pre-operatively		37.5 (37.2-38.1)	37.2 (37.0-37.6)	**0.02***

4.3.15 Pre-operative length of stay in hospital

The number of days of pre-operative stay in hospital was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" on a total of 316 patients as data was not available on 26 patients. The number of days of pre-operative stay in patients with histologically "normal" (median 1.0, IQ range 1.0-2.0) appendices was significantly increased compared with the acute (median 1.0, IQ range 0-1.0, p=0.006) appendicitis as well as with the complicated (median 1.0, IQ range 0-1.0, p=0.01) appendicitis patients. The number of days of pre-operative stay in patients with acute (median 1.0, IQ range 0-1.0) appendicitis did not vary significantly compared with complicated (median 1.0, IQ range 0-1.0, p=0.79) appendicitis (Table 4.32, graph 4.23).

Table 4.32: Summary of the analysis of the preoperative length of stay in hospital between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	112	1.0	0	1.0	1.0	0.01
II	108	1.0	0	1.0	1.0	
III	96	1.0	1.0	2.0	1.0	



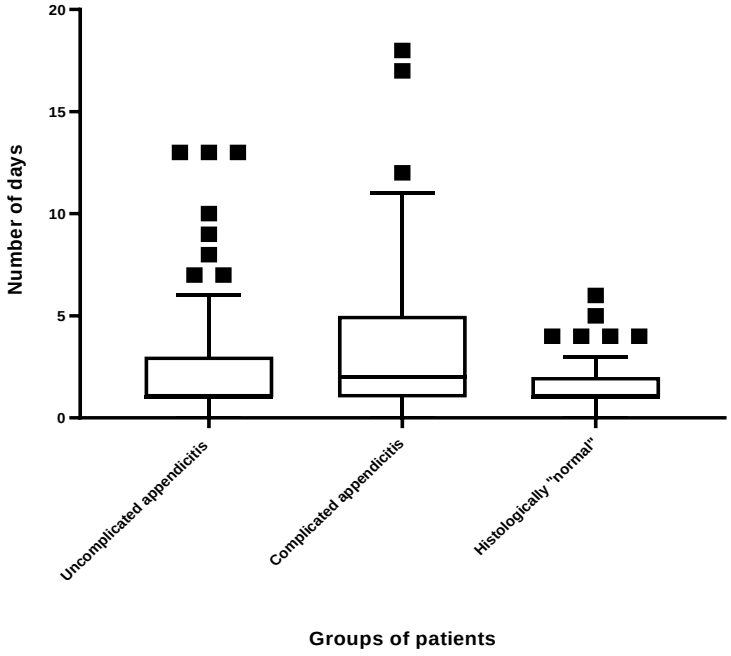
Graph 4.23: Tukey's boxplots displaying data of the pre-operative length of stay between the 3 groups of patients. Group I-patients uncomplicated appendicitis, median 1.0, 25th percentile (Q1) 0, 75th percentile (Q3) 1.0, interquartile (IQ) range 1.0; Group II-patients with complicated appendicitis, median 1.0, 25th percentile (Q1) 0.0, 75th percentile (Q3) 1.0, interquartile (IQ) range 1.0; Group III-patients with histologically "normal" appendices, median 1.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 2.0, interquartile (IQ) range 1.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

4.3.16 Post-operative length of stay in hospital

The number of days of post-operative stay was assessed in the groups of acute appendicitis, complicated appendicitis and histologically "normal" on a total of 312 patients as data was not available on 30 patients. The number of days of post-operative stay in patients with histologically "normal" (median 1.0, IQ range 1.0-2.0) appendices was significantly lower compared with the complicated (median 2.0, IQ range 1.0-5.0, $p<0.001$) appendicitis but did not differ significantly compared with the acute (median 1.0, IQ range 1.0-3.0, $p=0.06$) appendicitis patients. The number of days of post-operative stay in patients with complicated (median 2.0, IQ range 1.0-5.0) appendicitis was significantly increased compared with the acute (median 1.0, IQ range 1.0-3.0, $p=0.004$) appendicitis patients (Table 4.33, graph 4.24).

Table 4.33: Summary of the analysis of the postoperative length of stay in hospital between the 3 groups of patients. Group I-patients with uncomplicated appendicitis, Group II-patients with complicated appendicitis, Group III-patients with histologically "normal" appendices. N represents the number of patients that were included in the analysis. Data is presented with median, 25th percentile, 75th percentile and the interquartile (IQ) range. Statistically significant difference between the 3 groups was calculated using the Kruskal-Wallis H test. The results were considered as statistically significant when the probability value (p value) was less than 0.05.

Groups	N	Median	25th	75th	IQ range	p value
I	111	1.0	1.0	3.0	2.0	<0.001
II	105	2.0	1.0	5.0	4.0	
III	96	1.0	1.0	2.0	1.0	



Graph 4.24: Tukey's boxplots displaying data of the post-operative length of stay between the 3 groups of patients. Group I-patients uncomplicated appendicitis, median 1.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 3.0, interquartile (IQ) range 2.0; Group II-patients with complicated appendicitis, median 2.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 5.0, interquartile (IQ) range 4.0; Group III-patients with histologically "normal" appendices, median 1.0, 25th percentile (Q1) 1.0, 75th percentile (Q3) 2.0, interquartile (IQ) range 1.0. Median: line through the box, Q1: upper hinge, Q2: lower hinge, IQ range: difference between the upper (Q1) and lower (Q2) hinge (box), upper fence= 1.5 × IQ range, lower fence= Q1 - 1.5 × IQ range. Values plotted with ▪ represent outliers.

CHAPTER 5: Discussion

This study showed that different levels of expression of immune markers were well described with immunohistochemistry techniques in formalin-fixed and paraffin embedded appendix specimens. The pathology of the specimens varied significantly from complicated appendicitis (perforation or gangrene) to histologically "normal" samples resected from patients with suspected appendicitis in order to cover the entire inflammatory spectrum of the disease. The studied markers were chosen due to their key role in the inflammatory process as well as intestinal nociception. Their expression was assessed in the context of new evidence on their actions that has recently emerged. Our experimental findings were supported by clinical data on the same patients. This study provided clear evidence that there were profound differences in the clinical presentation of patients with different aspects of the disease.

The use of immunohistochemistry was decided as it is a robust tool for gaining more information on tissue specimens compared with the conventional histological examination. Immunohistochemistry examines protein expression and therefore provides information on the final product of a certain gene. A higher gene expression in a given situation does not always imply that the final protein will also be highly expressed. This is due to the RNA interference system that is able to silence gene expression at the mRNA level. Immunohistochemistry also has the advantage of demonstrating the location of the protein within the tissue architecture and it is more economical and convenient for clinical purposes compared with PCR [248-251]. Therefore, one could advocate that the use of immunohistochemistry was an

excellent and valid tool of examining the expression of inflammatory markers within the appendiceal tissue as provided information on protein expression combined with the location of the protein and it was easily reproducible and economical compared to gene analysis especially in a study with a large sample size such as this.

Despite the fact that acute appendicitis is the commonest surgical emergency, its diagnosis continues to be very challenging as, on one hand, it remains primarily clinical and, on the other, many patients present with atypical clinical picture. In addition to the diagnostic dilemma, the significant increase in morbidity and even mortality due to delay in treatment has led surgeons to perform early appendicectomy in patients with possible appendicitis. Although the introduction of this practice has resulted in a decrease of complications due to delays in the management of the patient, it did increase the number of histologically "normal" appendices removed from patients with a clinical picture of acute appendicitis. In the laparoscopic era, diagnostic laparoscopy has been employed to reduce the so called by clinicians "negative or unnecessary" appendicectomy. However, surgeons are often puzzled by the conflicting finding of a macroscopically normal appendix in a patient with strong clinical suspicion of appendicitis. Therefore, the dilemma of removing normal looking appendices from patients with right iliac fossa pain remains and the decision rests with the surgeon’s discretion in the absence of formal guidance or policy. In addition to the aforementioned clinical dilemma, the observation that appendicectomy relieves symptoms in most patients who subsequently found to have a histologically "normal" appendix may suggest the existence of an unknown pathology. This study has investigated the discrepancy between the clinical and

histological diagnosis and has shown that not only the degree of inflammatory response can vary significantly but also the histologically "normal" appendices removed from patients with right iliac fossa pain exhibit abnormal levels of cytokine expression.

The crucial role of serotonin in the pathogenesis of intestinal inflammation is widely acceptable [133, 161, 202, 205, 270, 271]. In this study, the serotonin contents of neuroendocrine cells were examined in appendices obtained from patients with clinical diagnosis of acute appendicitis. Immunostaining for serotonin revealed enterochromaffin cells in the crypts of the epithelium and subepithelial neuroendocrine cells in the *lamina propria* located close to the crypts in all four groups of the study. This study demonstrated that histologically proven inflamed appendices were noticeably depleted of serotonin in both the epithelium (enterochromaffin cells) and *lamina propria* (subepithelial neuroendocrine cells) when compared with histologically "normal" and control samples. Serotonin contents of the histologically "normal" appendices did not differ significantly from the control group. Our results were in agreement with the results reported by Vasei *et al.* [133] who also found that inflamed appendices were severely depleted of serotonin and histologically "normal" appendices from symptomatic appendices did not exhibit significant changes compared with the control group. However, our results and those reported by Vasei *et al.* [133] were in contrast with the results published by Dhillon *et al.* [152] who reported reduction in the serotonin immunostaining in subepithelial neuroendocrine cells and enterochromaffin cells of histologically "normal" appendices removed from symptomatic patients. Vasei *et al.*

[133], also investigated the serotonin contents of appendices with follicular hyperplasia and reported to be reduced compared with the control group. According to the authors, in the early stages of the inflammatory process such as follicular hyperplasia, serotonin excretion from neuroendocrine cells increases and might lead to histologically proven acute appendicitis.

In addition to the aforementioned findings, this study investigated the serotonin contents of specimens with complicated appendicitis (perforation or gangrene) which represents the most advanced stage of the inflammatory process. Interestingly, significantly less serotonin was observed in the neuroendocrine cells of appendices with perforation or gangrene compared with the uncomplicated samples. Given the fact that serotonin can act as a potent vasoconstrictor through 5-HT1 and 5-HT2β receptors [272], the finding of significantly reduced contents of serotonin in the neuroendocrine cells of patient with complicated acute appendicitis could imply that serotonin released by the ECC and SNCs in the interstitial tissue, could play an active role in the pathogenesis of ischaemia and subsequent necrosis or perforation of the appendiceal wall.

It is supported that enterochromaffin cells are able to sense the increased intraluminal pressure due to accumulated mucosal secretions following obstruction and respond by releasing serotonin [188, 273]. Serotonin is also a potent intestinal secretory mediator which further aggravates intraluminal secretions worsening the already increased intraluminal pressure [188]. However, according to the data presented in this thesis, 25% of the patients with complicated acute appendicitis

were found to have evidence of a faecolith within their appendix but the serotonin contents of neuroendocrine cells were not different between appendices with faecoliths and those without faecoliths. In a similar way, in the group of patients with acute uncomplicated appendicitis there was not any difference in the serotonin contents of appendices with and without the presence of faecolith within their lumen despite the fact that only 10% of patients with uncomplicated appendicitis had evidence of faecolith in their appendices. To the best of our knowledge there is not any previously published study to examine the serotonin contents in appendices with and without faecoliths in both complicated and uncomplicated appendicitis. The findings of this study could not support the theory according to which neuroendocrine cells respond to increased intraluminal pressure by releasing serotonin as the presence of faecolith within the appendiceal lumen did not change the observed levels of serotonin contained by the neuroendocrine cells.

Moreover, the reported interactions of serotonin with other inflammatory markers such as IL-1β, IL-6 and TNF-α, could also indicate that serotonin plays a central role in the pathogenesis of appendicitis. In the present study it was observed that the group of patients with complicated appendicitis who demonstrated to have neuroendocrine cells severely depleted of serotonin compared to patients with uncomplicated appendicitis were also found to have significantly increased epithelial IL-6 expression. However, the TNF-α expression between the two groups of patients did not differ significantly. Moreover, serotonin is known to enhance the phagocytic activity of macrophages as well as promote recruitment of inflammatory cells. This could potentially be reflected in the observation of significantly increased white cell

and neutrophil count in the blood of patients with complicated appendicitis compared with those with uncomplicated appendicitis. This work also demonstrated that patients with complicated appendicitis had significantly increased body temperature pre-operatively as well as increased white cell count and CRP. These findings could potentially be attributed to the enhanced expression of IL-6 due to increased interstitial serotonin, as IL-6 is a well-known pyrogen and is also able to promote the expression of acute phase proteins as well as proliferation of inflammatory cells [274-277]. On the other hand, in either group of complicated or uncomplicated acute appendicitis the serotonin contents of neuroendocrine cells did not seem to be directly linked to the presence of localised peritonism or previous episodes of right iliac fossa pain. Given the observed difference in serotonin contents between patients with complicated and uncomplicated appendicitis, as well as its ability to enhance the recruitment of inflammatory cells and the expression of potent pro-inflammatory mediators in combination with effective vasoconstriction [188, 273], it could be hypothesised that neuroendocrine cells and serotonin could be able to divert an insult to the appendiceal mucosa to a fully blown inflammatory process leading to necrosis.

With regards to histologically "normal" appendices and their serotonin contents in the enterochromaffin cells being similar to that of the control group, it could be hypothesised that the enterochromaffin cells of the appendiceal mucosa were not activated and, therefore, serotonin was not released into the bloodstream or interstitial tissues [92, 95, 192-194]. As serotonin is known to be one of the factors to promote recruitment of inflammatory cells, the unreleased serotonin in the

histologically "normal" appendices could be one of the reasons to explain the absence of neutrophil infiltration as it was evident on conventional microscopy. The stored serotonin in the enterochromaffin cells could not exhibit its vasoconstrictive action either [188, 273]. Moreover, since enterochromaffin cells are able to sense an increased intraluminal pressure, serotonin contents similar to normal could imply a normal intraluminal pressure. Interestingly, 26% of the histologically "normal" appendices had evidence of faecolith within their lumen. Thus, it could be speculated that either the appendicectomy was performed early and there was not enough time for the intraluminal pressure to rise or the presence of faecoliths was not enough to cause obstruction of the lumen which eventually would lead to the development of acute appendicitis. However, the clinical data of this work revealed that the duration symptoms in patients with histologically "normal" appendices was not statistically different from that of patients with inflamed samples and the pre-operative length of stay of patients with histologically "normal" appendices was significantly longer compared with that of patients with inflamed samples. Hence, one could advocate that in patients with histologically "normal" appendices, the appendicectomy was performed at a slightly later stage compared with patients with inflamed samples. As a result, the intraluminal obstruction should have been well established and the serotonin contents significantly reduced in patients with faecoliths. However, the difference in the serotonin contents between patients with and without faecolith was not statistically significant. Another interesting observation was that 25% of the patients with complicated appendicitis also had evidence of faecolith, however the findings on conventional microcopy were entirely different compared with those of patients with histologically "normal" appendices. Therefore, these findings could

imply that either the histologically "normal" appendices might not represent an early stage of acute appendicitis or the presence of faecolith could not be the initial stimulus that would eventually lead to acute appendicitis.

The serotonin contents of the subepithelial neuroendocrine cells were also examined in this thesis. Despite the fact that statistically notable differences were documented between the 4 different groups of patients, the number of subepithelial neuroendocrine cells was extremely small. Therefore, the statistical analysis might not have provided accurate results that could be used to explain the involvement of the subepithelial neuroendocrine cells in the pathogenesis of appendicitis. Nevertheless, the expression of interleukins with a key role in the intestinal inflammation and nociception was also investigated. Various levels of expression of TNF-α, IL-6 and IL-2R have been well demonstrated with immunohistochemistry.

The vast majority of cells stained by the anti-TNF-α antibody was macrophages located in the *lamina propria* of the appendiceal mucosa. The expression of TNF-α in specimens with histologically confirmed acute appendicitis, either complicated or uncomplicated was significantly higher compared with the control. The difference in TNF-α expression between samples with complicated and uncomplicated acute appendicitis was not statistically significant. The results of this thesis were in agreement with those reported by Wang *et al.* [129] who demonstrated intense cellular TNF-α mRNA expression in the germinal centres and moderate expression in the mucosa. The authors did not detect important differences between samples with suppurative and non-suppurative appendicitis. However, in this study, a significantly

increased TNF-α expression in the histologically "normal" specimens was observed compared with the acutely inflamed samples, whereas Wang *et al.* [129] reported that 7 of the 31 histologically "normal" appendices demonstrated similar TNF-α mRNA expression to the acute appendicitis specimens. On the other hand, in a study that involved 18 paediatric appendices, Murphy *et al.* [165], demonstrated that the gene expression of TNF in patients with acute appendicitis (mild or severe) did not vary significantly compared with the control samples. The study included only two histologically "normal" specimens. The discrepancy between the results of this thesis and those reported by Wang *et al.* and Murphy *et al.* could be attributed to the small number of patients involved in these studies. Wang *et al.* recruited 53 patients and Murphy *et al.* 18 patients whereas in this study TNF-α expression was assessed on 417 patients. However, it is appreciated that either gene analysis or *in situ* hybridisation using cDNA probes are time-consuming techniques involving complex protocols. Moreover, both studies were conducted on paediatric samples whereas the present study examined the expression of the inflammatory markers in adults. Despite the discrepancy in the results, both this study and that of Wang *et al.* demonstrated elevated levels of TNF-α in histologically "normal" appendices suggesting the presence of an inflammatory process. Another factor that could influence the outcome of each study and possibly explain the discrepancies between the studies was the different research method that was used in each study. In the present study, immunohistochemistry was chosen to assess the expression of different inflammatory mediators, and it differed significantly from gene expression or PCR as it assessed the final product of the gene expression. The higher expression

of a particular gene is not always linked to a higher protein level as gene expression can be silenced at mRNA level.

In a similar way, expression of IL-6 showed significant differences between the groups of the study. To the best of our knowledge, this was the first study to explore IL-6 expression in appendiceal tissue. It was observed that both epithelial and inflammatory cells of complicated appendicitis samples expressed significantly increased IL-6 compared with control and acute appendicitis samples. However, IL-6 expression between the control samples and those demonstrating features of acute appendicitis did not differ significantly for either epithelial or inflammatory cells. Interestingly, epithelial cells demonstrated significantly increased IL-6 expression in histologically "normal" appendices compared with the control and complicated appendicitis samples. On the other hand, non-epithelial IL-6 expression did not differ significantly from the complicated appendicitis group.

This study also demonstrated that different levels of IL-2R expression can be well described with immunohistochemistry. It was demonstrated that specimens with histologically confirmed appendicitis showed significantly increased IL-2R expression compared with histologically "normal" and control samples. However, complicated appendicitis samples expressed significantly more IL-2R compared with the non-complicated appendicitis specimens. Histologically "normal" appendices resected from patients with suspected appendicitis expressed significantly more IL-2R compared with the control samples but significantly less compared with the histologically confirmed inflamed specimens (either complicated or uncomplicated).

Wang *et al.* [129] investigated the expression of IL-2 in appendices using in-situ hybridisation. They reported that large numbers of IL-2 mRNA expressing cells were found in the *lamina propria* of all histologically confirmed appendices. This finding was in agreement with that of the present study, however, Wang *et al.* did not detect significant difference in the IL-2 mRNA expression between samples with suppurative and non-suppurative appendicitis. As for TNF-α, the authors reported that 7 of the 31 histologically "normal" appendices were found to express IL-2 mRNA similar to the acutely inflamed appendices. In this study IL-2R expression was notably increased in the histologically "normal" appendices but not as high as the acutely inflamed samples.

The finding of increased TNF-α, IL-6 and IL-2R in the histologically confirmed inflamed appendices could be easily explained as it has been shown that all these three markers play a crucial role in the acute inflammatory response [165, 221, 227-229, 278, 279]. Abnormal growth of intestinal bacteria causes damage to the epithelial barrier allowing increased bacterial translocation [280]. Lipopolysaccharides are important membrane components of the Gram negative bacteria and given the predominance of the Gram negative bacteria in the GI tract, lipopolysaccharides form primary targets for the immune system to recognise [228]. Bacterial endotoxins can trigger the innate immune response through communication with antigen presenting cells (macrophages and dendritic cells) [281]. Activated macrophages are known to produce IL-1β, a potent pro-inflammatory mediator, which is able to upregulate the expression of the very important pro-inflammatory cytokines such as TNF-α and IL-6, further promoting intestinal inflammation [282-286]. Moreover, antigen presenting

cells of the intestinal *lamina propria* are capable of presenting antigens to B and T lymphocytes leading to activation of the adaptive immunity [281]. Once T lymphocyte activation is facilitated by its antigen receptor, IL-2 is rapidly synthesised [281, 287]. This is succeeded by expression of the IL-2 receptor, hence the IL-2/IL-2R interaction promotes quick proliferation of the activated T lymphocytes [288] . IL-2 along with IL-15 also stimulates proliferation of natural killer cells and together with IL-12 enhances their cytotoxic activity [289-292]. In a similar way to T lymphocytes, IL-2 stimulates expression of IL-2R in B lymphocytes, further enhancing their sensitivity to IL-2, promoting antibody secretion from the activated B lymphocytes [293, 294]. Therefore, IL-2 could act synergistically with other cytokines in order to affect various cells types for the optimal immune response to be achieved.

The finding of increased TNF-α and IL-6 expression in the complicated appendicitis samples compared with the uncomplicated acute appendicitis samples could reflect a much more prolonged inflammatory status due to the fact that it was not treated early. However, clinical data on patients included in this study did not demonstrate significant difference in the duration of the symptoms between patients with uncomplicated and complicated acute appendicitis. Therefore, complicated acute appendicitis could also represent a much more severe inflammatory response suggesting a different immunopathogenesis between complicated and uncomplicated appendicitis.

The striking finding of significantly increased IL-6 expression in the specimens with complicated appendicitis compared with uncomplicated appendicitis samples could

be one of the factors to explain the difference in severity of the inflammatory process between the two entities [295-299]. It has been shown that IL-6 is a potent pro-coagulant cytokine stimulating tissue factor (TF) mRNA expression [278]. As a result, it could be suggested that increased IL-6 expression could promote local thrombosis which could subsequently lead to tissue hypoxia and eventually to gangrene and/or perforation of the appendiceal wall. Increased IL-6 expression has been linked with the severity of the inflammatory process as De Hooge *et al.* [299] reported that homozygosity for the IL-6 -174 G-allele was responsible for increased risk of severe juvenile arthritis in humans. Moreover, Rivera-Chavez *et al.* [297] proposed that the IL-6-174 single nucleotide polymorphisms (SNPs) could enhance the expression of IL-6, which in turn could contribute to the severity of acute appendicitis. Ruber *et al.* [295, 300] detected increased levels of the pro-inflammatory cytokines IL-6 and IL-17 in the plasma of patients with gangrenous appendicitis. On the other hand, Murphy *et al.* [165] demonstrated that the expression of IL-17 was very low in the mild and severe acute appendicitis patients. Therefore, although there is strong evidence to support that appendicitis is not characterised by Th1 or Th2 immune response, the evidence regarding Th17 immune response is conflicting and there is need for further research.

TNF-α has also been shown to be a potent pro-inflammatory mediator that has been linked with the severity of the inflammatory response. In patients of Hispanic origin TNF-α-308 A-allele has been associated to complicated appendicitis but not to uncomplicated [229]. TNF-α can also promote local thrombosis and subsequent necrosis as it has been demonstrated to promote Tissue factor (TF) expression and

decrease the expression of TF expression inhibitors [301]. However, the differences in the expression of TNF-α between patients with complicated and uncomplicated acute appendicitis were not statistically significant. Therefore, TNF-α in acute appendicitis could act synergistically with other cytokines and might not be as crucial as that of IL-6 in the development of complicated acute appendicitis.

The clinical data of this study also provided substantial evidence of the severity of the inflammatory process in patients with complicated appendicitis. The patients of this group were found to have significantly higher body temperature in the pre-operative period. Moreover, their white cell count and CRP were also significantly higher compared to patients with uncomplicated appendicitis. As discussed earlier, the high body temperature could be attributed to the increased expression of IL-6, which is a well-known pyrogen [274]. IL-6 is also able to promote the expression of acute face proteins as well as proliferation of inflammatory cells [274, 275, 277, 302, 303]. Interestingly, the expression of IL-6 in the group of uncomplicated acute appendicitis was very similar to that of the control samples as it did not differ statistically. Another, striking finding was that the duration of the symptoms between patients with complicated and uncomplicate acute appendicitis did not vary significantly. Moreover, the severity of pain was found to be significantly higher in patients with complicated appendicitis but the presence of localised peritonism did not differ between the two groups of patients. On the other hand, previous episodes of right iliac fossa pain were only reported by 4% of the patients with complicated appendicitis whereas 14% of the patients with uncomplicated appendicitis reported previous right iliac fossa pain. In none of the two groups of patients with previous

episodes of right iliac fossa pain or presence of localised peritonism the expression of the studied inflammatory mediators or any of the clinical parameters was affected. Finally, a faecolith was found in 25% of patients with complicated appendicitis and in only 10% of patients with uncomplicated appendicitis which was statistically significant. Interestingly, the presence of faecolith in patients with either complicated or uncomplicated acute appendicitis did not affect the expression of any of the studied inflammatory markers or the clinical parameter that were examined. The results of this study on the clinical data between the two groups of patients could outline some fundamental differences in the presentation as well as the severity of the immune response in each of the two entities of the inflammatory spectrum of acute appendicitis.

The finding of IL-6 expression being significantly higher in the complicated acute appendicitis samples compared to those with uncomplicated appendicitis and the IL-6 expression of the uncomplicated acute appendicitis samples being very similar to that of the normal samples, combined with the clinical data that showed no difference in the duration of symptoms to suggest acute appendicitis could possibly indicate that some aspects of the immunopathogenesis of the two entities are different. This could be further supported by the findings on the severity of pain which was significantly higher in the complicated acute appendicitis patients and the presence of faecolith which was significantly higher in patients with complicated appendicitis. Therefore, complicated appendicitis could not just be the progression of an inflammatory process that was left untreated but an entity with different immunopathogenesis from that of uncomplicated acute appendicitis.

This concept could be supported by an epidemiological study which revealed that complicated and uncomplicated acute appendicitis have followed completely different epidemiological trends from 1970 to 2004 [279]. Complicated appendicitis showed a slow increase in incidence, whereas uncomplicated appendicitis followed a U-shaped curve. Therefore, if complicated appendicitis was the consequence of delayed management, the trends of the two entities should have been almost parallel during the course of the period that they were studied [279].

On the other hand, given the fact that not only the expression of IL-6, but also the expression of TNF-α and IL-2R, was significantly increased in both groups of histologically proven acute appendicitis compared to normal tissues, it could also be hypothesised that a uniform inflammatory response between complicated and uncomplicated acute appendicitis could be present, but at some crucial point during the inflammatory response the expression of key inflammatory markers could either be selectively upregulated or downregulated to the extent that tissue ischaemia ensues which will eventually lead to perforation or gangrene. The presence of faecolith within the appendiceal lumen could be one of the factors to lead to necrosis. However, the presence of faecolith did not affect the expression of any of the studied inflammatory markers or clinical parameters within each group of patients.

Murphy *et al.* [165] provided some evidence to support this theory. The gene profiles that the researchers studied appeared to be very similar in both mild and severe appendicitis with the vast majority of the genes that were differentially expressed in the mild group, were also differentially expressed in the severe appendicitis group.

Therefore, it was demonstrated that a core set of genes was activated at all samples irrespective of the degree of inflammation suggesting that there was a uniform inflammatory response underlying the pathogenesis of appendicitis. However, despite the fact that Murphy *et al.* [165] were able to demonstrate high expression of IL-1 which is a potent proinflammatory mediator, there was no detectable induction of TNF.

In this study, although the expression of TNF-α was significantly increased compared with the control samples it did not differ compared with samples that had evidence of gangrene or perforation. These findings could indicate that despite the very similar histological findings in different degrees of inflammation in the acute appendicitis spectrum, the actual inflammatory response might be a highly specific process with the activation of different inflammatory pathways. However, further research is required to identify potential stimuli that would steer the inflammatory process towards gangrene or perforation. Although obstruction of the appendiceal lumen followed by bacterial overgrowth, increased luminal pressure and subsequent tissue ischaemia and eventually necrosis, is generally considered as one of the triggering factors of the disease, there is limited amount of evidence to support or refute this theory. The current study demonstrated that 25% of the patients with complicated appendicitis had evidence of faecolith in their appendices compared to 10% of patients with uncomplicated appendicitis, but neither the expression of the studied inflammatory markers nor the clinical parameters that were investigated varied significantly between patients with or without faecoliths. Murphy *et al.* [165]

provided similar results as the cytokine expression did not differ between patients with and without faecoliths.

Based on the data provided by the current study combined with the very limited previously published evidence, one could advocate that the immunopathogenesis of complicated appendicitis could be at least partly different form that of uncomplicated appendicitis and not just progression of the disease. It could also be supported that specific inflammatory markers might be differentially regulated and eventually tissue ischaemia ensues. Moreover, the presence of faecolith within the appendiceal mucosa might not be such an important factor that could lead to ischaemia as it has been thought to be. However, further research is required to strengthen or dispute this theory.

This study also provided new evidence regarding the group of histologically "normal" appendices resected from patients with a diagnosis of acute appendicitis as it was demonstrated that all the studied cytokine markers were notably increased in this group of patients. More specifically, the TNF-α expression in the histologically "normal" appendices was significantly increased compared with the normal as well as histologically proven inflamed appendices. The histologically "normal" appendices also demonstrated significantly increased IL-6 expression in the epithelial cells compared with the control and the inflamed samples, either complicated or uncomplicated. The mucosal and submucosa IL-2R expression of the histologically "normal" specimens was significantly increased compared to the control appendices but it did not reach the levels of the expression observed in the inflamed samples.

The increased expression of both TNF-α and IL-6 which have been shown to be potent pro-inflammatory cytokines could suggest the potential disruption of the integrity of the appendiceal mucosa and the subsequent evasion of bacterial pathogens such as lipopolysaccharides. This would lead to activation of the host defense response as part of the innate immunity. This finding taken together with the increased expression of IL-2R which is expressed at a slightly latter stage as described previously, could suggest a fully activated immune system. The presence of a fully activated immune system would give rise to signs and symptoms of varying severity to suggest an ongoing inflammatory response. Indeed, this study demonstrated that patients who were subsequently found to have histologically "normal" appendices presented with the clinical picture of acute appendicitis. Interestingly, the severity of pain as well as the presence of nausea/vomiting in patients with histologically "normal" appendices did not vary significantly compared with patients with inflamed appendices. More than half of the patients with histologically "normal" appendices also had features of peritonism on clinical examination. Therefore, according to the data of this thesis, the clinical presentation of patients with a subsequently histologically "normal" appendix was very similar to that of patients with histologically confirmed acute appendicitis which could also be due to an activated immune system.

The findings of this study on the expression of the studied inflammatory markers could indicate the presence of an inflammatory response at such an early stage where there was no evidence of neutrophilic infiltration, which is the hallmark of acute inflammation on conventional microscopy. This could be supported by the

observation that although IL-2R expression was increased, as it is involved in regulating the immune response by promoting lymphocyte proliferation, it did not reach as high levels as of that observed in the inflamed samples. The results on the clinical data could further support this, as the neutrophil count in patients with histologically "normal" appendices apart from being significantly lower compared to that in the group of patients with inflamed samples, it was very similar to that of the control group. Moreover, the lymphocyte count in the group of patients with histologically "normal" appendices was significantly higher compared with any of the other group of patients.

It could also be speculated that TNF-α and IL-6 expression was at higher levels during the very early stages of the inflammatory process when the antigen presentation took place. This could explain the interesting finding of increased TNF-α and epithelial cell IL-6 expression in the histologically "normal" appendices compared with the histologically proven acutely inflamed samples. The presence of faecolith in 27% of the histologically "normal" samples could be at least partially in agreement with this as the complicated appendices also featured faecoliths in 28% and the inflammatory cell expression of IL-6 between the histologically "normal" and complicated appendicitis samples did not differ. These findings could indicate that the initial insult to the appendiceal mucosa by the increased intraluminal pressure due to faecolith and the subsequent bacterial overgrowth caused a significant rise in the expression of both TNF-α and IL-6 as both cytokines have been shown to be part of the innate immune system and actively participate in the antigen presentation [304, 305].

The theory that histologically "normal" appendices resected from symptomatic patients could represent an inflammatory response at a very early stage could be supported by the findings of Murphy *et al.* [165] who reported changes in inflammatory gene expression before cellular infiltration was present in the appendiceal mucosa. Therefore, the findings of this work on the studied cytokines as well as the clinical data, taken together with the existing evidence on gene expression, could suggest that most of the appendices with an inflammatory process evident only at a molecular level, might progress to microscopically typical appendicitis if left untreated. On the other hand, such an early inflammatory process could possibly resolve spontaneously.

The caveat of this theory was that it could not explain several aspects of the clinical presentation of patients with histologically "normal" appendices. An inflammatory response at a very early stage in combination with absence of structural damage to any of the anatomical layers of the appendiceal wall in patients with histologically "normal" appendices would be extremely difficult to give rise to abdominal pain of such severity as of that reported by patients with perforated appendicitis. Moreover, it would be very challenging to explain why the duration of the symptoms before patients with histologically "normal" appendices presented themselves to the medical team was very similar to those with histologically proven inflamed samples. However, the most intriguing finding that this theory could not really explain was the presence of previous episodes of right iliac fossa pain to a significant proportion of patients with histologically "normal" appendices. It was found that 23% of patients with histologically "normal" appendices experienced several episodes of right iliac

fossa pain prior to admission whereas that proportion was 4% and 14% of patients with complicated and uncomplicated acute appendicitis respectively. Furthermore, a very early inflammatory response that could potentially develop into a microscopically evident acute inflammation could not justify a high lymphocyte count as this is an indication of a chronic inflammatory status. Finally, according to the data presented in this thesis on the presence of faecolith, the proportion of patients with faecolith and histologically "normal" appendices was not different to that of patients with complicated appendicitis. This finding taken into consideration with the very similar duration of symptoms between the different groups of patients, one could raise the question why the early inflammatory response did not develop into microscopically evident inflammation. Therefore, the interpretation that histologically "normal" appendices resected from symptomatic patients could exhibit features of such an early inflammatory status that was only evident at a molecular level could not account for several aspects of the clinical presentation of those patients. Finally, another factor that could refute this theory was that, immunohistochemistry, in contrary to gene analysis, assessed the final product of gene expression that was biologically active within the tissue and not just the expression of certain genes that could be muted at some point downstream. Thus, the findings of this study could suggest that the level of the inflammatory process in histologically "normal" appendices was beyond molecular level.

Contrary to the widely accepted perception according to which the initial response to local infection is pro-inflammatory, recent evidence suggested that many features of the acute phase response could be anti-inflammatory as suppression of the

inflammatory response has been shown to be beneficial for the host [296]. Rivera-Chavez *et al.* [296] described the cytokine profile in the peritoneal fluid and plasma of patients with histologically proven appendicitis and demonstrated that peritoneal response was characterised by increased expression of both pro- and anti-inflammatory cytokines whereas the plasma (systemic) cytokine profile appeared to be primarily anti-inflammatory.

As mentioned in the introduction, IL-6 is not only an important pro-inflammatory mediator but it can also exhibit anti-inflammatory properties in both local and systemic anti-inflammatory responses [223]. It has been shown that during endotoxemia the absence of IL-6 resulted in a significantly higher circulating level of pro-inflammatory cytokines such as MIP-2, IFN-γ and TNF-α as these cytokines have been implicated in the elicitation of toxic syndromes [223]. This was further supported by the finding that IL-6 was able to restrict recruitment of neutrophils promoting their replacement by mononuclear cells steering the inflammatory response towards chronicity [229]. Evidence suggested that this transition was directed by IL-6 *trans* signalling [227, 228].

Moreover, there is evidence to support that TNF-α also has anti-inflammatory properties [221, 280]. Noti *et al.* [221] reported that, during acute intestinal inflammation, TNF-α promoted the release of intestinal glucocorticoids which in turn can control the activation of intestinal T lymphocytes. Furthermore, TNF-α has been shown to induce apoptosis of immune and non-immune cells [306, 307]. Although TNF-α-induced apoptosis of T lymphocytes was shown to help terminate or control

intestinal inflammation, apoptosis of epithelial cells compromised the integrity of the intestinal barrier even further allowing constant activation of intestinal immune cells with luminal antigens [217, 306].

This could be supported by the finding of elevated IL-2R expression in the histologically "normal" appendices, as IL-2 expression is transient and depends on sustained antigenic stimulation [238]. IL-2 and IL-2R are known to be crucial for the maintenance of immune homeostasis as shown in studies using IL-2 and IL-2R knockout mice. IL-2 apart from playing a significant role in the expansion of activated lymphocytes it has been shown to be a vital cytokine in downregulating immune responses [240, 308]. Moreover, studies have demonstrated that a major function of the IL-2/IL-2R system is associated with the development and function of T regulatory cells [309].

Therefore, one could advocate that the markedly increased expression of IL-6 and TNF-α in the histologically "normal" appendices could indicate an anti-inflammatory profile of the immune response. The anti-inflammatory response could aim to suppress an either abnormally long or abnormally regulated inflammatory response. IL-2 could act synergistically with IL-6 and TNF-α as it is vital in the homeostasis of the immune response [47, 240, 310]. On the other hand, the fact that TNF-α could potentially compromise the integrity of the intestinal mucosa [217], it would lead to a vicious cycle of repeated inflammation, due to prolonged exposure to bacterial LPS. Consequently, several attempts in resolving an inflammatory response could lead to a chronic inflammatory status.

The clinical data of the current study appeared to support this theory. First and foremost, the presence of previous episodes of right iliac fossa pain in patients with histologically "normal" appendices was significantly increased compared to patients with inflamed appendices. However, on the acute presentation of the patient to the medical team, neither the severity of the pain nor the duration of symptoms in patients with histologically "normal" samples varied from those with histologically proven inflamed samples. This piece of clinical data could suggest that patients with histologically "normal" appendices were subjected to either a recurrent inflammatory stimulus or the initial inflammatory response never managed to successfully treat the initial stimulus. In keeping with a chronic or abnormally long inflammatory response was the finding of a significantly increased lymphocyte count in patients with histologically "normal" appendices. In a similar way, patients with histologically "normal" samples exhibited a significantly low neutrophil to lymphocyte ratio. Moreover, although the white cell count of patients with histologically "normal" appendices was increased compared with the control group, it did not reach the levels observed in patients with histologically proven appendicitis, either uncomplicated or complicated. Interestingly, patients with histologically "normal" appendices had significantly low levels of C-reactive protein compared with the patients of the control as well as the histologically proven appendicitis groups. It is acknowledged that C-reactive protein is not a specific marker and the presence of malignancy could cause a significant rise in the C-reactive protein concentrations. Therefore, the C-reactive protein might not be a reliable inflammatory marker to allow for valid conclusions as in the current study the comparison was between inflamed samples due to bacterial antigen and malignant cells.

With regards to signs suggesting acute appendicitis, 56% of patients with histologically "normal" appendices were found to have evidence of peritonism compared to 73% of the patients with either uncomplicated or complicated appendicitis. This finding highlighted the fact that patients with subsequently histologically "normal" appendices did present with features of acute appendicitis. As a result, although it was very challenging to assess the degree of inflammation, appendicectomy could have been the correct decision from a patient safety point of view. It could also indicate that, despite the absence of microscopic evidence of inflammation, the severity of symptoms during the acute presentation to the surgical team and subsequent admission to hospital, were such that appendicectomy was clinically justified.

Another feature that could be linked to a prolonged inflammatory response was the surprisingly high proportion of patients with histologically "normal" appendices that were found to have faecoliths. It could be speculated that faecoliths could cause intermittent or temporary obstruction of the appendiceal lumen giving rise to abdominal pain. It could also be suspected that the abdominal symptoms could be relieved when the distal pressure in the appendiceal lumen reached the necessary levels to expel the faecolith. Therefore, repeated cycles of obstruction could cause recurrent episodes of abdominal pain. The recurrent episodes of inflammation could compromise the function as well the integrity of the appendiceal mucosa leading to a vicious cycle of inflammation, due to entry of bacterial LPS, and tissue repair. This theory could be compatible with the clinical finding of recurrent episodes of abdominal pain in a large proportion of patients with histologically "normal"

appendices. Therefore, the chronic antigenic stimulation of the immune system could lead to an abnormally regulated inflammatory response that would exhibit anti-inflammatory features in order to prevent harmful effects to the host. As a result, it could be supported that the fact that some appendices remained histologically "normal" having minimal consequences to the host could be due to the increased levels of anti-inflammatory cytokines such as IL-6 and TNF-α that downregulated the inflammatory response.

The interpretation that increased levels of IL-6 as part of an anti-inflammatory response in order to protect the host was supported by the findings of Xing *et al.* who demonstrated that local and systemic IL-6 could modulate an pro-inflammatory cytokine profile in various tissues [311]. Apart from another study by Xing *et al.* [223] which demonstrated that IL-6 knockout mice suffered an increased mortality following administration of a lethal dose of endotoxin, IL-6 has also been shown to exhibit similar action in chronic diseases in an attempt to elicit cellular immune responses as well as humoral responses against re-infection [312-315].

The experimental data of this study taken in combination with the clinical data could possibly suggest that the immune response of the patients with subsequently histologically "normal" appendices displayed some anti-inflammatory features. This could be based on the fact that both TNF-α and IL-6 can exhibit potent anti-inflammatory actions. In conjunction with raised IL-2R expression, these cytokines could aim to control an abnormally long or abnormally regulated inflammatory response. The role of IL-6, although very controversial could be of paramount

importance as it has been shown to steer the inflammatory response to chronicity and inhibit the recruitment of neutrophils [221, 227-229, 240, 307, 308]. These findings combined with the clinical finding or significantly increased previous episodes of right iliac fossa pain in patients with histologically "normal" appendices as well as the increased lymphocyte count and the simultaneous absence of neutrophils in the tissue could indicate an abnormally regulated chronic inflammatory response. On the other hand, the fact that the expression of different inflammatory mediators as well as the clinical parameters between patients with faecolith and those without faecolith did not differ significantly, is difficult to explain.

It is appreciated that the current study provided experimental data on the expression of four inflammatory markers and clinical parameters originated from a single centre. In addition to this, there is not enough published data to take into consideration the differential action of several inflammatory cytokines as well as genes and their role in the inflammatory profile of the different aspects of appendicitis. Therefore, conclusions based on the existing evidence might not be robust and further research on this topic is of paramount importance.

Cytokines have also been considered to be important hyperalgesic mediators and, therefore, a major cause of abdominal pain in patients with intestinal inflammation [288, 316, 317]. Thus, the finding of increased interleukin expression in the group of patients with histologically "normal" appendices could be of significant clinical relevance. Several studies have suggested that both TNF-α and IL-6 have significant influence in nociception [289-293]. The experimental findings of this work were

supported by clinical data as it was apparent that there were significant findings to suggest that patients with subsequently histologically "normal" appendices had genuine abdominal pain which was very similar to the pain that was experienced by patients who had histologically proven acute appendicitis. First and foremost, the severity of pain experienced by patients with histologically "normal" appendices did not differ significantly from that of patients with histologically proven samples. In addition to this, the duration of pain immediately prior to the acute presentation did not differ between patients with histologically "normal" and inflamed samples. Interestingly, the pre-operative length of stay of patients with histologically "normal" appendices was significantly longer compared with patients who had complicated or uncomplicated appendicitis. One could suspect that this was due to the fact that the surgical team was not convinced about the underlying pathology and subsequently the diagnosis. The association of the right iliac fossa pain with evidence of neutrophilia in a young patient is highly suggestive of appendicitis. Therefore, the absence of neutrophilia along with low C-reactive protein concentrations in patients with subsequently histologically "normal" appendices might have puzzled the surgical team who decided to observe the patient for a longer period. Another baffling finding was the presence of localised peritonism in 56% of patients with histologically "normal" appendices. The absence of peritonism in almost half of these patients might have also contributed to the longer pre-operative period for observation purposes. On the other hand, it was very difficult to explain the presence of localised peritonism in patients without any anatomical damage to the layers of their appendices. Interestingly, the expression of the studied inflammatory markers did

not vary between patients with peritonism and those without within the group of patients with histologically "normal" appendices.

The argument that at least some of the symptoms suggesting appendicitis could be attributed to the increased expression of the studied cytokines could be supported by several studies. Ibeakanma and Vanner [316] reported that supernatant from biopsies taken from patients with ulcerative colitis induced hyperexcitability of nociceptive colonic neurons which was mediated by TNF-α. Hughes *et al.* [288] demonstrated that both TNF-α and IL-6 were increased in supernatants from IBS patients with diarrhoea as the main symptom (D-IBS). The receptors of TNF-α, IL-6 and IL-1β that were located on colonic afferent neurons were able to sensitise colonic afferents and therefore, they were linked to the abdominal pain experienced by the patients with D-IBD [288]. In a study conducted by Humes *et al.* [317], patients with symptomatic diverticular disease were shown to have increased expression of TNF-α and IL-6 which was associated with visceral hypersensitivity to rectal balloon distension. The aforementioned studies provided strong evidence that TNF-α and IL-6 were able to induce hyperalgesia when their expression was upregulated during an inflammatory response in the colon. Therefore, the symptom of right iliac fossa pain suggesting acute appendicitis in patients with subsequent histologically "normal" appendices could be attributed to increased levels of TNF-α and IL-6. However, the absence of other signs and symptoms to complete the classical clinical picture of acute appendicitis could possibly explain the decision made by the surgical team to observe the patients for a longer period.

However, it is suspected that the clinical picture of patients with a subsequent histologically "normal" appendix during most of their recurrent admissions would not convince the surgical team to proceed with appendicectomy. Thus, these patients would either be discharged or treated conservatively with antibiotics. A Cochrane review showed that selected patients could be managed conservatively with antibiotics [318]. However, the authors concluded that appendicectomy should remain the standard treatment. Three major randomised controlled trials have supported antibiotic treatment over appendicectomy although the appendicectomy rates in patients treated with antibiotics varied from 24% to 48% [319-321]. The conservative management as treatment of appendicitis has not been supported by the findings of the present study. Faecoliths have been strongly associated with complicated appendicitis for which conservative management is contraindicated. In a very recent and well-designed randomised control trial although the authors were in favor of antibiotic treatment over appendicectomy, 39.1% of the patients who were treated with antibiotics needed appendicectomy within the 5-year follow-up period [322]. The study included adult patients with CT-proven uncomplicated appendicitis only. Interestingly, patients with evidence faecoliths on the CT scan were excluded [322]. Therefore, one could advocate that the option of conservative management in patients who have been suspected to have appendicitis, but the clinical picture does not suggest severe inflammation to warrant emergency appendicectomy, might not be effective due to the presence of faecoliths within the appendiceal lumen. This could be of significant importance in countries that either do not have the infrastructure to perform CT scan for every patient with suspected

appendicitis or CT scans are not recommended due to the young age of most patients who are suspected to have appendicitis.

Moreover, even in the laparoscopic era where laparoscopy can be used as a diagnostic tool, there is not a clear policy or guidance to recommend the resection of a macroscopically normal appendix during laparoscopy. Some studies have concluded against the removal of a macroscopically normal appendix due to the associated risks [126]. However, as faecoliths have been associated with complicated appendicitis, either the early resection of an appendix or the resection of a macroscopically normal appendix might prevent a later admission with complicated appendicitis. Indeed, the randomised control trial by Salminen *et al.* reported that two out of the thirty patients who were treated conservatively with antibiotics and needed appendicectomy in the 5-year follow-up period, were found to have complicated appendicitis [322]. However, none of the two patients had evidence of faecoliths on the CT scan at the time of randomisation. Interestingly, another two patients from the same group that needed appendicectomy in the 5-year follow-up period were reported to have histologically normal appendices. The latter implied that these two patients presented with a clinical picture of acute appendicitis that convinced the surgical team to perform appendectomy despite the fact that conventional microscopy did not demonstrate any evidence of an inflammatory response. In the same study, the overall complication rate was 24.4% in the appendicectomy group and 6.5% in the antibiotic group. However, all the patients who required appendicectomy underwent an open procedure according to the study

design. Nevertheless, it is known that laparoscopic appendicectomy is associated with significantly less complications [323].

Another significant finding of the present study and of paramount importance in the laparoscopic era was that almost half of the histologically "normal" appendices appeared to be inflamed intraoperatively. This finding highlighted that the clinical judgement of the severity of inflammation cannot be always trusted. Interestingly, the cytokine levels between the histologically "normal" appendices that appeared inflamed and those that appeared normal did not demonstrate significant deference strengthening the postulation that the intraoperative diagnosis is not reliable even further. Therefore, one could support that patients could present with either a classical picture of acute appendicitis and undergo a laparoscopic appendicectomy or their signs and symptoms would not be convincing of acute appendicitis and following a brief period of observation they would undergo diagnostic laparoscopy where their appendix could appear macroscopically normal. These appendices could even be histologically "normal" albeit they could still exhibit abnormal levels of cytokines responsible for the symptomatology. Therefore, there could be a case to remove macroscopically normal appendices in patients undergoing laparoscopy for right iliac fossa pain.

In conclusion, to the best of our knowledge this is the first study to use experimental data from the entire spectrum of appendicitis in conjunction with clinical data as well as results from routine laboratory tests and intraoperative findings in order to further explore the pathogenesis of appendicitis. It provided clear evidence that

histologically "normal" appendices resected from patients with right iliac fossa pain with a subsequent clinical impression of acute appendicitis exhibited abnormally elevated levels of cytokine expression. Moreover, the current study showed that patients with a subsequent histologically "normal" appendix could present with signs and symptoms suggesting acute appendicitis. It also described the expression of key inflammatory markers and clinical presentation of patients with different inflammatory states of the spectrum of appendicitis.

The data of this study suggested that histologically "normal" appendices resected from symptomatic patients exhibited features of an activated immune system. As discussed earlier, this activation could represent either an inflammatory response at very early stages that could not be detected on conventional histopathologic examination or it could represent an attempt to resolve an inflammatory response the duration of which was abnormally long. The effect of that could be an anti-inflammatory profile of the immune response.

Finally, the difference in cytokine expression between complicated and non-complicated appendicitis and the pathological cytokine expression in histologically normal appendices could suggest a different immunopathogenesis in these diseases and not just disease progression. As mentioned earlier in the discussion, this was supported by existing evidence on the epidemiology of complicated and uncomplicated appendicitis [279]. This was further supported by the clinical findings presented in this thesis. Interestingly, despite the fact that were no differences in the duration of symptoms, clinical findings such as body temperature, white cell count,

neutrophil count and C-reactive protein were significantly elevated in patients with complicated appendicitis. Therefore, it could be speculated that the spectrum of appendicitis could consist of entities with different pathogenesis rather than variations in the degrees of inflammation.

CHAPTER 6: Conclusion

The findings of the present study have established that histologically "normal" appendices resected following a clinical impression of acute appendicitis due to right iliac fossa pain exhibit abnormally elevated levels of cytokine expression. It has also been demonstrated that immunohistochemistry was able to describe significant differences in the level of expression of the studied cytokines between different degrees of inflammation in appendices resected from symptomatic patients. Moreover, immunohistochemistry was able to detect increased levels of cytokine expression at a stage where inflammatory infiltrate was not detectable with conventional histologic examination. Therefore, the use of immunohistochemistry in testing histologically "normal" samples for altered cytokine expression could supplement the clinical findings and convert a "negative or unnecessary" appendicectomy to a clinically justifiable one.

The work presented in this thesis has documented new and not previously published findings. To the best of our knowledge, this is the first study to present experimental findings on cytokine expression in conjunction with clinical data from signs and symptoms as well as routine laboratory investigations and intraoperative findings from patients with appendicitis covering the entire spectrum of the disease.

With regards to the experimental findings it was reported that in histologically "normal" appendices: ***(1)*** both TNF-α and epithelial IL-6 expression were significantly increased compared with the control as well as the inflamed appendices; ***(2)*** both mucosal and submucosal expression of IL-2R were significantly increased compared

with the control samples but not as high as in the inflamed appendices; ***(3)*** serotonin contents of enterochromaffin and subepithelial neuroendocrine cells did not demonstrate significant difference compared with control samples.

The uncomplicated acute appendicitis samples in comparison with the complicated appendicitis specimens demonstrated that: ***(1)*** serotonin contents were significantly higher in both enterochromaffin and subepithelial neuroendocrine cells; ***(2)*** TNF-α expression did not differ; ***(3)*** IL-6 expression in both epithelial and inflammatory cells were significantly lower and ***(4)*** IL-2R levels in both mucosa and submucosa were also significantly lower.

As far as the clinical data was concerned, patients with histologically "normal" appendices in comparison with those with histologically confirmed inflamed samples demonstrated that: ***(1)*** previous episodes of right iliac fossa pain were significantly increased; ***(2)*** the duration as well as the severity of abdominal pain did not differ; ***(3)*** localised peritonism was evident in 56%; ***(4)*** the body temperature on admission did not differ; ***(5)*** the white cell and neutrophil counts were significantly reduced; ***(6)*** the lymphocyte count as well as the neutrophil to lymphocyte ratio were significantly increased; ***(7)*** the C-reactive protein concentrations were significantly reduced; ***(8)*** the preoperative length of stay was significantly longer and ***(9)*** faecoliths were evident in 27%. Interestingly, the expression of the studied inflammatory markers as well as the clinical parameters did not differ between patients with faecolith and those without faecolith in any of the three groups. Regarding the intraoperative diagnosis, ***(10)*** 51% of the histologically "normal" appendices appeared to be

inflamed intraoperatively. However, the expression of the studied inflammatory markers as well as the clinical parameters did not vary between the appendices that appeared normal and those that appeared inflamed.

The results of this work suggested that histologically "normal" appendices resected from symptomatic patients exhibit features of an activated immune system. This activation could be due to an inflammatory response at very early stages that was not detectable on routine histopathologic examination or an attempt to resolve an abnormally long inflammatory response. The latter could imply an immune response with anti-inflammatory features and beneficial effects to the host. This was supported by the clinical findings of this work as well as published evidence to demonstrate that TNF-α, IL-6 and IL-2 can also function as anti-inflammatory mediators.

The fundamental differences in cytokine expression between complicated and non-complicated appendicitis could suggest a different immunopathogenesis in these entities and not just disease progression. This was supported by previously reported data on the epidemiology of complicated and uncomplicated appendicitis as well as clinical data presented in this thesis. Moreover, given the increased expression of the studied cytokines in the histologically "normal" appendices one could advocate that the different degrees of inflammation of appendicitis could not be due to progression of an inflammatory process that has been left untreated. A better understanding in the expression of inflammatory genes as well as the expression of their final product

such as cytokines, interferons, and receptors is essential for enlightening several aspects of the immunopathogenesis of appendicitis.

CHAPTER 7: Limitations and future studies

7.1 Limitations of the present study

It is appreciated that this study had several limitations. First and foremost, it was a single centre study. Although the inclusion criteria were as broad as possible in order for the study population to be as close as possible to the true population of patients who were diagnosed with acute appendicitis, the experimental data originated from just one hospital, the Queen's Medical Centre in Nottingham in the UK. Despite the fact that it is a large tertiary centre, the number of patients who seek medical advice due to right iliac fossa pain could vary significantly between different areas of the UK Moreover, the pathogenesis of appendicitis could be significantly different in different populations. Genetic factors as well as environmental factors implicated in the pathophysiology of acute appendicitis could also differ significantly in patients from different geographical locations. Apart from possible variances between different patient populations, there might be several hospital factors that could differ between different countries or even hospitals within the UK that could influence the inflammatory response and consequently the experimental data. Characteristic example of this, could be the time that the decision to operate was made. As a result, the expression of inflammatory mediators in the tissue could vary significantly. In addition to this, the use of antibiotics as treatment for appendicitis might be another factor that could potentially alter the inflammatory response. Therefore, in hospitals where the use of antibiotics is the first line of treatment for appendicitis, the *in-situ* expression of inflammatory could be significantly different. Thus, as the experimental data in the present study originated from patients who were treated in a single centre, the conclusions of this study might not apply in different patient populations.

Another limitation of the present study was the retrospective nature of it. With regards to the clinical data that was collected, the parameters that steered the diagnosis towards acute appendicitis, were already documented in patients notes. The critical issue with this, was that the study design could not account for certain clinical/laboratory tests to be performed at a specific time in the diagnostic process. It was attempted to collect data from the day of admission as described in the methodology. However, that was not the case for all the patients in the study and therefore some clinical data on some patients was missing. Moreover, the clinical data that was used in this study was recorded by different members of the surgical team with different levels of experience. Although, the collected data derived from entries of the most senior members of the team, even then the levels of seniority did vary and that could have influenced some of the results. Typical example of this was the documentation of the clinical examination findings and more specifically the presence of peritonism. However, the study was conducted in a University Hospital that tends to attract senior registrars who already had several years of surgical experience. In addition to that, as it was conducted in a tertiary centre the consultants were very much involved in patient's care in order to achieve the best possible outcome. Regarding the actual experimental work, the retrospective nature of the study did not allow to formulate even more robust inclusion and exclusion criteria that could be applied before the appendicectomy took place. In a prospective study, the members of the research team could be part of the team that admitted the patients, had active involvement in the formulation of the clinical diagnosis as well as in the administration of the intervention in order to eliminate heterogeneity. However, a prospective study with a large study population would have been

significantly more time consuming and would not be possible to be completed in the duration of a doctoral course. Moreover, in the given timeframe a prospective study would have resulted in a significantly smaller study population. Hence, the present study included a large number of patients, which was significantly larger compared to any of the previously published studies, but did not allow the members of the research team to be directly involved in the clinical aspects of patient management peri-operatively in order to control for some cofounding factors and produce even more robust results.

Another limitation of this work was the nature of the specimens that formed the control group. The vast majority of the control samples was obtained from patients with underlying colonic malignancy. Therefore, the samples might not be entirely normal, and the expression of the studied inflammatory mediators could be different from that of completely healthy tissues. Although patients with obstructing colonic tumours, caecal tumours or with evidence of inflammatory bowel disease were excluded, the presence of malignancy is known to be accompanied by a notable systemic inflammatory response that could potentially affect the levels of several inflammatory mediators even when the tissue appears normal on conventional microscopy. On the other hand, one could easily understand that it was impossible to find totally healthy appendix specimens for this type of study. Moreover, the mean age of the patients who formed the control group was significantly different from that of the rest of the study population. It was apparent that this was due to the underlying pathology of the control group as colorectal malignancy is prevalent in older patients. The inflammatory response in older patients could be significantly

different from that in younger and therefore the expression of the studied inflammatory mediators could have been different in healthy individuals of younger age. However, it was extremely difficult to obtain a significant number of appendix samples, as the study design dictated, from either entirely healthy or with benign diseases other than inflammatory bowel disease young individuals.

Finally, although the use of immunohistochemistry is a robust way to assess expression of inflammatory mediators; a fact that is evident from the number of previously published studies that used immunohistochemistry, it could also be considered as a limitation of this study as it is a mostly semi-quantitative method. The H-scoring system is a widely used method to assess the immunostaining and is formulated by two components as described in Chapter 3. As the first component is the percentage of the positively stained cells and not the actual number of the positively stained cells, it renders the H-score a semi-quantitative method of assessing the *in-situ* levels of the studied markers. Therefore, the calculated expression of inflammatory mediators is not the same as the actual one but a very close estimate. The use of this scoring method, however, can be very helpful in sections where the number of positively stained cells is extremely large and as a result the process of counting all the positively cells is close to impossible. The second component of the H-score is the intensity of the immunostaining which is represented by a number (1 for weak, 2 for medium and 3 for strong). Different groups of cells found in different areas in the section may have different intensity of immunostaining. The issue with this component of the scoring system lies with the subjectivity of the intensity between different researchers. This is the reason why in

most published studies either all or a representative and predetermined number of sections have been scored by two different researchers and as soon as the two scores are within an accepted range the score is finalised. In this study, immunohistochemistry for four inflammatory mediators was performed. In order to overcome the inherent problem of subjectivity as well as semi-quantification of the immunostaining it was decided to quantify the expression of two (TNF-α and IL-2R) out of the four markers by painstakingly counting the positively stained cells. The *in-situ* levels of serotonin were simultaneously assessed by the author of this thesis and a senior Histopathology consultant, Dr A. M. Zaitoun. Both researchers had to agree on the score given to each slide. The expression of IL-6 was semi-quantified by the author of this thesis using the H-scoring system. 25% of the slides were independently assessed by Dr A. M. Zaitoun. As the differences in the scores between the two researchers were not significant, the scores obtained by the author of this thesis were used in the analysis. Another way to obtain quantitative data is the use of more modern techniques such as the qPCR to assess mRNA expression. However, such techniques are much more expensive compared to immunohistochemistry and their use in the present study was prohibited due to the limited budget. Moreover, fluorescent immunohistochemistry in combination with confocal microscopy would add great value to this study. Despite some of the known challenges associated with this technique such as the high background signal, it could help to investigate the exact distribution of the studied inflammatory markers and more specifically their possible co-localisation and how this could differ across the entire spectrum of appendicitis. On the other hand, the low cost and ease of reproducibility of

immunohistochemistry allowed for a large study population that was significantly larger compared with any of the previously published studies.

7.2 Future studies

Acute appendicitis is the most common surgical emergency and can present as an inflammatory spectrum. However, the pathogenesis of appendicitis and the reason why it presents as a spectrum remains unknown. Further and deeper understanding of the inflammatory response in patients with appendicitis is crucial. Elucidation of the differences in the immune responses that take place in the different aspects of the appendicitis spectrum would help clinicians tailor their approach to patients with appendicitis. Therefore, complications due to either a delayed surgical intervention in patients who could not be managed conservatively or exposure of to an unnecessary operation in patients that could be treated conservatively, can be avoided.

First and foremost, a large prospective study is needed that can overcome some of the limitations of the present study. In such study the research team would be able to recruit patients based on their signs and symptoms upon their presentation and not on their Histological diagnosis that was made post-operatively. This method would enable researchers to directly interact with patients in order to not only diminish the issue of missing data but also minimise heterogeneity. The prospective design of the study would also enable the researchers to account for factors that can alter the inflammatory response such as administration of antibiotics. Very often, patients with either atypical symptoms of appendicitis or very early stages of appendicitis are initiated on antibiotics and only referred to the surgical team when the abdominal pain is not settling, or the symptomatology of appendicitis becomes more apparent. To the best of our knowledge, there is not any published study on

appendicitis that compared the *in-situ* expression of inflammatory mediators in patient who were treated with antibiotics pre-operatively and those who were taken to theatre without prior administration of antibiotics. Therefore, a prospective study design could be very helpful in assessing the effect of antibiotic treatment in the pre-operative period in relation to the underlying histopathological features of appendicitis as well as clinical features such as the presence of faecoliths in the appendiceal lumen.

Extension of this work could include documentation of the expression of other cytokines (such as IL-1, IL-4, IL-5, IL-8, IL-12, IL-17 and IFN-γ) that are centrally involved in the immune response to antigenic stimuli. Even though appendicitis is much more common in young adults compared to inflammatory bowel disease, the type of inflammatory response is not known. Traditionally, the inflammatory response in ulcerative colitis was thought to be an atypical type 2 (Th2) response characterised by expression of IL-5 but not IL-4 whereas, Crohn's disease has been associated with type 1 (Th1) cell mediated inflammatory response including the expression of IL-12, IFN-γ and TNF-α. However, more recently, evidence of increased expression of IL-17 in patients with ulcerative colitis has emerged and therefore, ulcerative colitis could be associated with Th17 inflammatory response. Moreover, increased expression of IL-8 has been observed in the intestinal mucosa of patients with either ulcerative colitis or Crohn's disease. IL-8 is also known as a neutrophil chemotractant interleukin and its expression has been linked with acute appendicitis. However, the expression of several key cytokines in the inflammatory response has either been examined in a very small number of samples or not been examined at all.

More specifically, the expression of different cytokines in histologically "normal" appendices remains very poorly investigated. Thus, one could easily understand that extensive research is required to fully characterise the inflammatory response in acute appendicitis. Immunohistochemistry can be very useful in describing the expression of several inflammatory markers in a large number of patients and across the entire spectrum of appendicitis. Additionally, immunohistochemistry can be combined with immunofluorescence for better understand of the distribution of each cytokine as well as the co-localisation of the studied inflammatory markers across the entire range of appendicitis.

Nevertheless, further and better understanding of the gene expression in acute appendicitis as well as histologically "normal" appendices resected from symptomatic patients is crucial. Acute appendicitis is characterised by the infiltration of neutrophils into the mucosa of the appendix. However, in the case of altered gene expression, whether the observed changes are the result of alterations in the gene expression of the resident cells within the appendix or they reflect the genes expressed by the infiltrating neutrophils needs to be thoroughly investigated. The events that might occur in the earliest stages of inflammation could lead to important findings regarding the pathogenesis of appendicitis. Detailed documentation of the gene expression in histologically "normal" appendices could establish whether the observed alterations took place prior to the infiltration of neutrophils or not. Ultimately, delineation of the pathogenesis of this high prevalent disease could lead to more accurate diagnosis and eventually influence the treatment.

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Appendix

1 IHC protocol

Solutions and reagents

1. Neutral-buffered formalin, 10% (NBF), 1 Liter

- Distilled H_2O 900ml
- Di-sodium hydrogen phosphate, anhydrous (Na_2HPO_4) 6.5 grams
- Sodium di-hydrogen phosphate, monohydrate (NaH_2PO_4. H_2O) 4.0 grams
- Formaldehyde, 37% solution 100ml
- Adjust pH to about 7.0 with 1M (Molar: 1mol/L) NaOH or 1M HCL
- Final concentrations:
    - Formaldehyde 3.7%
    - Na_2HPO_4 mM
    - NaH_2PO_4. H_2O 29 mM

2. 10x Citrate Buffer stock, 1 liter

- Sodium hydroxide 10 g
- Citric acid 21 g
- De-ionised water 1000 ml
- Store at 4 °C in glass container

3. 1x Citrate Buffer working solution, pH 6.0

- Dilute 1 part of 10x stock solution with 9 parts of de-ionised water
- Adjust pH to 6.0 using 1M NaOH or 1M HCL as required

- Use within 24 hours

4. 10x Tris Buffer Stock (TBS) pH 7.6

- Trizma base 6.04 g
- Sodium Chloride 81 g
- Hydrochloric acid 1M 36ml
- Final volume (1L) using de-ionised water
- Adjust pH to 7.6 using 1M HCL if needed

5. 1x TBS working solution

- Dilute 1 part of 10x TBS stock solution with 9 of parts de-ionised water
- Adjust pH to 7.6 using with 1M NaOH if necessary
- Store working solution at room temperature and use within two weeks

6. Primary antibody detection system

- The Novolink™ Polymer Detection System will be used for visualisation of the primary antibody. The reagents of this detection system are:
    - Peroxidase Block: 3-4% (v/v) Hydrogen peroxidase
    - Protein Block: 0.4% Casein in phosphate-buffered saline, with stabilisers, surfactant and 0.2% Bronidox L as a preservative
    - Post Primary: rabbit anti mouse IgG (<10 µg/ml) in 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950
    - Novolink™ Polymer: anti-rabbit Poly-HRP-IgG (<25µg/ml) containing 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950

- DAB Chromogen: 1.74% w/v 3,3' – diaminobenzidine, in a stabiliser solution
- Novolink™ DAB Substrate Buffer (Polymer): buffered solution containing ≤0.1% hydrogen peroxide and preservative
- Haematoxylin: <0.1% Haematoxylin

(Source: www.LeicaBiosystems.com)

2 IHC procedure

1. Tissue preparation

- Fix tissue in 10% neutral buffered formalin for at least 24 hours. Embed tissue in paraffin wax according to embedding machine manufacturer's instructions. Formalin has a very slow diffusion coefficient, so the tissue needs to be no more than 1 cm thick. This procedure has already been done by the NHS staff. The initial tissue blocks will be melted, and two tissue specimens will be re-embedded into a new tissue block which will be labelled appropriately.

2. Tissue sectioning

- Prepare 5 µm sections on the microtome and place on clean, positively charged microscope slides
- Heat in tissue-drying oven for 45 minutes at 60°C

3. De-paraffinisation

- Place slides on the 60°C hotplate for 10 minutes
- Wash slides 2 times for 5 minutes in xylene

4. Rehydration

- Wash slides 3 times for 2 minutes in Industrial Methylated Spirit (IMS)
- Wash slides for 2 minutes in de-ionised water
- Wash slides for 5 minutes in running water

5. Antigen retrieval

- Heat slides in 1x citrate buffer working solution, pH 6.0 at 95-100°C for 20 minutes (microwave oven: Whirlpool JT359 6th sense)
- Cool slides with running water for 5 minutes

6. Immunostaining (Novolink™ procedure)

- Apply 100 µl Peroxidase for 5 minutes
- Wash slides in TBS 2 times for 5 minutes
- Apply 100 µl Protein Block for 5 minutes
- Wash slides in TBS 2 times for 5 minutes
- Apply 100 µl of primary antibody (optimally diluted in Leica Bond™ antibody diluents) and incubate for required time
- Wash slides in TBS 2 times for 5 minutes
- Apply 100 µl Post Primary Block for 30 minutes
- Wash slides in TBS 2 times for 5 minutes

- Apply 100 µl Novolink™ Polymer for 30 minutes
- Make up DAB working solution; 1:20 DAB Chromogen in Novolink™ DAB Substrate Buffer
- Wash slides in TBS 2 times for 5 minutes
- Apply 100 µl DAB working solution for 5 minutes
- Wash slides in TBS 2 times for 5 minutes
- Apply 100 µl Haematoxylin for 6 minutes
- Rinse slides in tap water for 5 minutes

7. Dehydrate and mount slides

- Wash slides 3 times for 2 minutes in IMS
- Wash slides 2 times for 5 minutes in xylene
- Mount with mounting medium
- Apply coverslip

(Source: www.LeicaBiosystems.com)