Corticotropin-releasing factor and acute post-operative gut function in traumatic abdominal injury and elective abdominal surgery

Lauren Hill

BSc (Med) Hons Nutrition and Dietetics

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Department of Human Biology

University of Cape Town

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Poetry is in the breath and finer spirit of all knowledge; it is the impassioned expression which is in the countenance of all Science.

William Wordsworth

Abstract

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Shocked trauma patients in the Intensive Care Unit undergo a powerful, neuro-endocrine stress response driven by cytokine release and the hypothalamic-pituitary-adrenal axis. The response is activated under stress by corticotropin-releasing factor (CRF), the well-known 41 amino acid peptide neuro-hormone. Evidence from animal and human studies suggests that peripheral CRF is present in the gastrointestinal tract and associated with inflammatory changes. Critically ill patients frequently display somewhat unexplained gastrointestinal dysfunction including delayed gastric emptying, ileus and increased bowel permeability. The aim of the study was to investigate the role of CRF in critically ill adults with traumatic abdominal injury compared with elective surgical patients, and describe any association of CRF levels with alterations in acute post-operative gastrointestinal function**.**

Eight patients with haemorrhagic shock following penetrating abdominal injury and seventeen patients undergoing elective surgery for hepato-biliary disease were studied for serial plasma and intestinal tissue CRF levels using radio-immunoassay. A RT-PCR technique was used to detect mRNA for CRF in intestinal tissue. Light microscopy was used to determine the quantity and distribution of mast cells in intestinal tissue. Post-operative gastric emptying was assessed using the paracetamol absorption test and intestinal permeability by measuring urinary lactulose:mannitol ratios following a bolus of these sugars. The study was approved by University of Cape Town Human Research Ethics Committee. Informed consent (retrospectively in the case of the trauma patients), was obtained from all subjects.

Circulating plasma levels of CRF were significantly increased in the shocked patients compared to the elective surgery patients at all time points, but both groups had levels of CRF which were higher than the reference values. CRF peptide was detectable in intestinal tissue at similar levels in both trauma and elective surgery subjects. CRF mRNA was not detected in intestinal tissue. Mast cell infiltration was increased, particularly in the mucosal and muscularis layers of the trauma subjects. Intestinal permeability was increased and gastric emptying was delayed in all subjects, and the latter was related to morphine dose.

Corticotropin-releasing factor is present in human small intestine during elective surgery and emergency surgery for haemorrhagic shock but there is no evidence of local synthesis. Plasma CRF levels are increased by surgery and particularly haemorrhagic shock. Intestinal inflammatory changes occur early after shock, but gastrointestinal dysfunction is similar following elective and emergency trauma surgery. The role of peripheral CRF in the gastrointestinal dysfunction seen in critically ill patients remains uncertain and requires further investigation.

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Abbreviations and Symbols

°C	degrees Celsius
ACTH	adrenocorticotropic hormone
ANS	Autonomic nervous system
APACHE	Acute Physiology and Chronic Health Evaluation
ATP	adenosine triphosphate
AUC	area under the curve
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
C18	carbon 18
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
cm^3	cubic centimetre
C_{max}	maximum concentration
cpm	counts per minute
CRF	corticotropin-releasing factor
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EPI	epinephrine
g	centrifugal force
g	gram
GAPDH	glyceraldehyde phosphate dehydrogenase
gDNA	genomic DNA
GIT	gastrointestinal tract
GUS1B	glucuronidase 1B
HPA	hypothalamic-pituitary-adrenal
HPLC	high pressure liquid chromatography
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
ICU	Intensive Care Unit
IgA	Immunoglobulin A
IgG	immunoglobulin G
ISS	Injury Severity Score
IQR	interquartile range
l	litre
LC-NE	Locus coeruleus-norepinephrine
M	Molar
mg	milligram

min	minutes
ml	millilitre
mm^2	square millimetre
mm^3	cubic millimetre
mM	millimolar
MOD	Multiple Organ Dysfunction
MOF	Multiple Organ Failure
mRNA	messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NE	norepinephrine
ng	nanogram
pg	picogram
PCR	polymerase chain reaction
pmol	picomol
RIA	radio-immunoassay
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
SIRS	Systemic inflammatory response syndrome
µg	micrograms
µl	microlitre
TAE	Tris/acetate/EDTA
Taq	*Thermus aquaticus*
TBE	Tris/borate/EDTA
TIU	trypsin inhibitor units
T_{max}	Time at which C_{max} occurs
U	units
V	volts

DNA nucleotide bases

A	adenine
T	thymine
C	cytosine
G	guanine

Symbols

α	alpha
β	beta
γ	gamma
µ	micro

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Introduction

Gastrointestinal dysfunction is a widespread problem in the critically ill patient. Delayed gastric emptying, abnormal motility patterns, and impaired intestinal barrier integrity are commonly observed in the Intensive Care Unit particularly in patients with traumatic injury and shock (Heyland et al, 1996; Kao et al, 1998; Montejo, 1999; Ritz et al, 2000). Impaired gut function compromises delivery of enteral nutrition, and is associated with complications and morbidities, which may impact upon survival (Ziegler et al, 1988; Mentec et al, 2001; Gramlich et al, 2004). Since successful enteral feeding is an important determinant of clinical outcome, a functional gut in the critically ill trauma patient is of considerable importance and clinical relevance. Multiple clinical processes and interventions could account for the disrupted gastrointestinal function in the critically ill. The actions of many physiological mediators also undoubtedly impact upon gastrointestinal function in a manner that is apparently extremely complex. Among such substances is corticotrophin-releasing factor (CRF).

CRF is a 41-amino acid peptide neuro-hormone first described by Vale et al in 1981. As a central molecule in the ancient stress response system it is widespread in the animal kingdom. CRF is the well-known hypothalamic effector of the physiological stress reaction. It is expressed and copiously secreted by the secretory neurons of the hypothalamic paraventriculum. When released into the hypophyseal portal vessels it impinges upon the pituitary, inducing the release of ACTH. Thus, CRF operates as the biological fulcrum of stress activation and regulation.

Besides its established role as the releasing-factor which drives the HPA axis, more recent data has shown that CRF is of importance in peripheral tissues as well as in the brain. In rats, CRF mRNA and/or peptide have been detected in various non-hypothalamic regions of the brain (Merchenthaler et al, 1982; Turnbull and Rivier, 1999) as well as in the thymus, spleen, heart, spinal cord, adrenal gland and inflammatory sites (Baigent and Lowry, 2000; Thompson et al, 1987; Mastorakos et al, 1995). Human skin (Lytinas et al, 2003), inflamed synovial membranes (Uzuki et al, 2001; Crofford et al 1993) and immune cells (Stephanou et al 1990; Ekman et al, 1993) also synthesise and release CRF. Furthermore, both rodent (van Tol et al, 1996; La Fleur et al, 2005) and human gut (Kawahito et al, 1995; Muromatsu et al, 2000) have been demonstrated to express CRF.

The presence of the stress hormone, CRF, in the gastrointestinal tract is of particular interest, because stress itself induces a typical pattern of functional changes to the gut. These include slowed gastric emptying, colonic motile stimulation, and impairment of the intestinal epithelial barrier. An accumulation of data, especially from animal studies, indicates that CRF is central to these stress-related alterations in gastrointestinal physiology. This occurs via both central and peripheral mechanisms involving CRF receptors in the gastrointestinal tract.

The importance of CRF in human gastrointestinal dysfunction is far from well defined, although it seems probable that it has a major role. Several points of interest on this topic prompted this study. These were:

- There is a paucity of direct evidence of a role for CRF in gastrointestinal dysfunction in humans. Further studies in man are therefore necessary.
- There is a lack of definitive data to explain the mechanism underlying the typical gastrointestinal dysfunction observed in the critically ill.
- There is a similarity between the gastrointestinal dysfunction associated with critical illness, and that reportedly associated with CRF activity. This raises the question of whether CRF is in any way involved in the gastrointestinal dysfunction of critically ill patients.

This study was therefore conceived in order to interrogate these issues. After a brief recap of the basic physiology of stress, the relevant supporting literature will be reviewed and the hypothesis of the study introduced.

Chapter 1: Literature Review

1.1. The physiological response to stress

The physiological response to stress is activated and modulated via the CRF-locus-coeruleus-norepinephrine (LC-NE) system, the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal circuits. Basal activity in these systems in the resting body serves to maintain homeostasis in the organism. Heightened activity initiated by threatening/destabilising stress causes these systems to respond with a co-ordinated neuroendocrine adaptive response intended to ensure survival. This response is typified by changes in metabolism, cardiovascular function and immune competence (Chrousos, 1995; Chrousos, 1992a).

The stress response (see Figure 1.1.) is effected by hypothalamic release of corticotropin-releasing factor (CRF), which is the main stimulus to the pituitary, and causes release of adrenocorticotropic hormone (ACTH). This in turn has a steriogenic effect on the cortex of the adrenal glands, stimulating release of glucocorticoids (primarily cortisol) into the circulation.

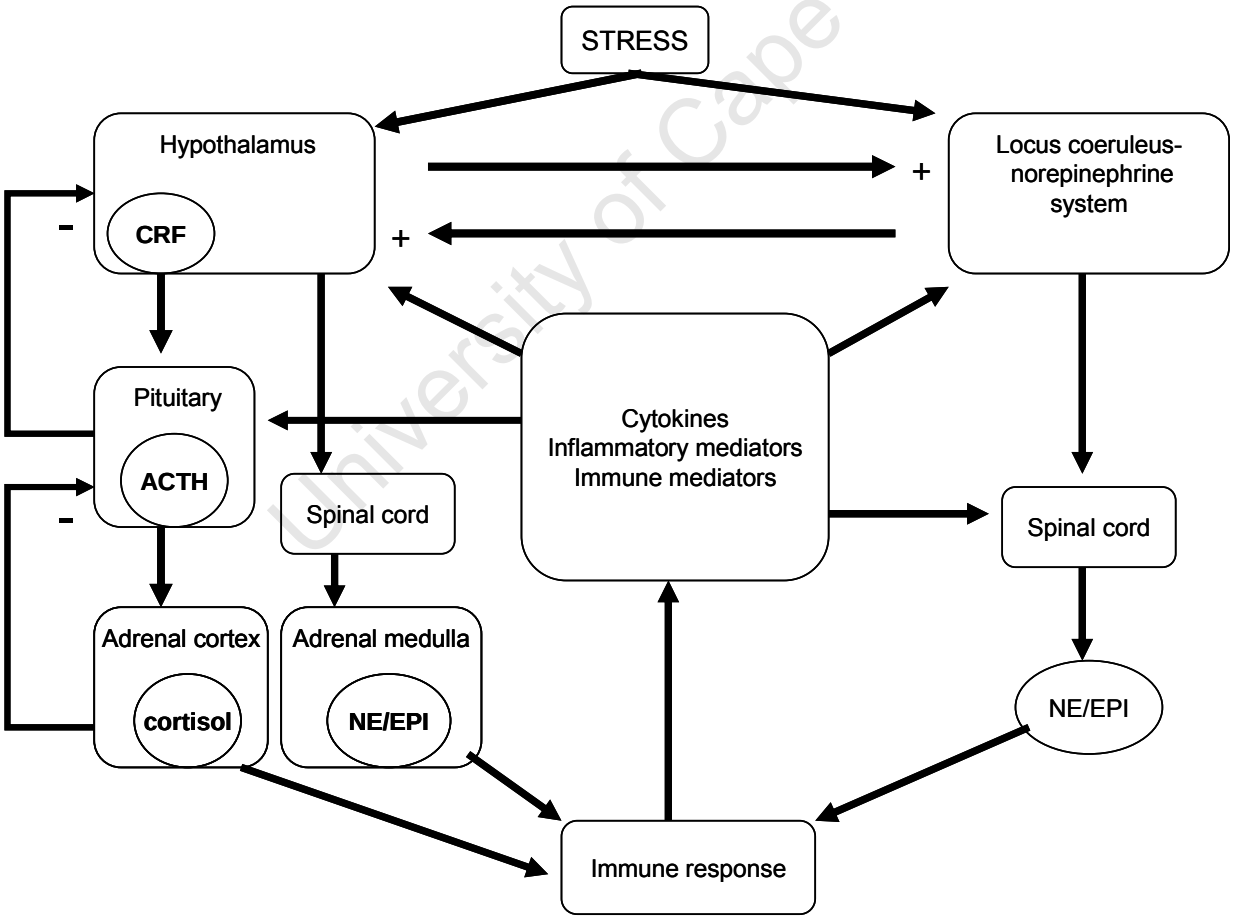


Figure 1.1. Integrated stress response

CRF corticotropin-releasing factor, ACTH adrenocorticotropic hormone, NE norepinephrine, EPI epinephrine

Adapted from Elenkov and Chrousos, 1999 and Mayer and Collins, 2002.

About 95% of the cortisol in the blood circulation is bound to carrier proteins, and is therefore not biologically active. The remaining 5% of blood cortisol is free in solution, enabling it to pass through cellular membranes, bind to receptors in the cytosol which translocate to the nucleus, resulting in increased gene transcription or inhibition via nuclear receptors such as nuclear factor kappa B. These effects can generally be described as catabolic, anti-inflammatory and immunosuppressive, and are very well characterised and widely known. Specific metabolic effects of cortisol include hepatic gluconeogenesis, glycogenolysis, skeletal muscle degradation and reduced muscle protein synthesis. Cortisol opposes insulin action, which results in decreased glucose uptake in both muscle and adipose tissue. In the cardiovascular system cortisol enhances vascular reactivity, probably via a permissive action on catecholamines. It also acts on lymphoid tissue to increase the ratio of neutrophils to eosinophils and basophils, at the same time causing an increase in red cell and platelet numbers. Additionally, at high doses glucocorticoids have anti- inflammatory and immunosuppressive effects, which are not seen in physiological concentrations. In large amounts glucocorticoids affect virtually every aspect of the physiological inflammatory-immune response – vasodilatation, capillary permeability, phagocytosis, antibody production, and lymphocyte number and lifespan. Hyper-cortisolism and deranged pituitary adrenal axis function in the critically ill elicited as a response to overwhelming physical stress, is associated with poor outcome, indicating how fine a balance is required in this system for homeostasis to continue (van den Berghe, 2002).

As depicted in Figure 1.1. the second arm of the integrated stress response is driven by the sympathetic nervous system. Centrally, the two systems (neuronal and endocrine) involved in the integrated stress response operate in a positive feedback loop through CRF-sympathetic cross-talk (Figure 1.2.) (Saper et al, 1976; Calogero et al, 1988; Chrousos and Gold, 1992; Chrousos, 1992b; Dunn and Swiergel, 2008).

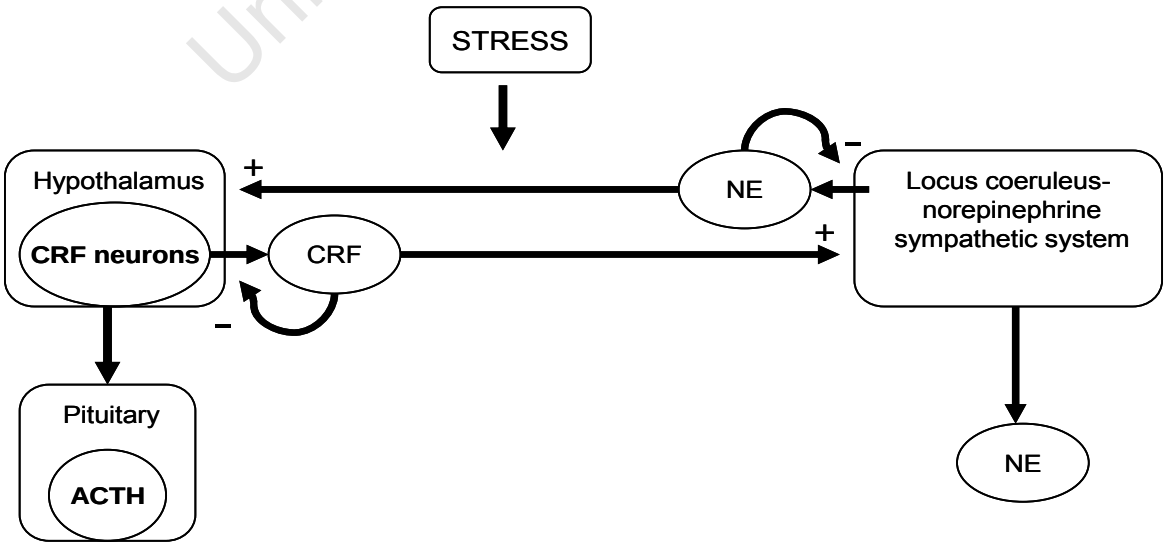


Figure 1.2. Control mechanisms modulating corticotrophin-releasing factor secretion

CRF corticotropin releasing factor, ACTH adrenocorticotropic hormone, NE norepinephrine. Adapted from Chrousos, 1992a

In brief, the mechanism involves the release of central CRF under the stimulus of norepinephrine. CRF neurons acting on α-noradrenergic receptors also stimulate norepinephrine secretion from the locus coeruleus. In this way, the release of CRF is reinforced by norepinephrine and vice versa. Negative feedback controls on this reciprocal reinforcement loop are glucocorticosteroids, ACTH, and cortisol, as well as ultra-short negative feedback loops of CRF on itself at hypothalamic level, and norepinephrine on itself at locus coeruleus level.

The endocrine and neural responses are only two parts of a much more complex system which is beyond the scope of this review. However, it is important to note two further aspects. Firstly, the central network integrates significant limbic input from other brain structures as well as from afferent visceral pathways (Mayer, 2000; Sawchenko et al, 2000). This is important because emotional stress and emotional outflow, processed centrally by the limbic system, can have profound effects on end organs under conditions of stress. In particular, the gut is influenced in this way, just as the HPA axis is an endocrine arm of the brain-gut axis. Secondly, many cytokines, inflammatory and immune mediators, neuro-active substances and hormones are also able to influence the stress response (Chrousos, 1995). Not only does the influence of such molecules occur at many different levels, but they additionally exert simultaneous influence on each other via a series of physiological feedback loops. This makes the entire system extremely complex. It is this very complexity that allows true brain-body interactions to occur, since no organ system operates in isolation. The remainder of this review will attempt to discuss the brain-gut axis as one such interaction by outlining the known effects of stress on the gastrointestinal system.

1.1.1. Critical injury as a stressor

In the healthy organism stress is intended to be a warning and therefore a protective mechanism. However, the stress response in a severely destabilised individual can also have negative effects or induce pathophysiological alterations. Critical injury can be thought of as the “ultimate example of acute, severe physical stress” (van den Berghe, 2002). The critically ill patient exhibits a profound physiological stress response, which is in proportion to the degree of injury or illness. Such patients require medical intervention to support vital organs so as to prevent inevitable death.

Critically ill patients are recognised to be a heterogeneous group. Patients enter the Intensive Care Unit (ICU) with various different injuries, underlying medical conditions, surgical procedures, and with different reasons for requiring critical care. Patients suffering a traumatic, life-threatening injury are suddenly and simultaneously exposed to multiple acute stressors including the physiological stress of the injury itself, haemorrhage, shock, pain, fear and anxiety. This may be followed by fluid therapy (including blood product transfusion), anaesthesia, surgery, and subsequent complications including sepsis. However, the stress response is apparently largely unaffected by the type of stressor, and thus clinical and physiological manifestations of stress are similar among different patient groups.

It appears that the stress response may be particularly harmful if coupled with inflammatory stimuli or events (Collins, 2001). Under such circumstances the physiological response may itself be more threatening than the original pathological event. The Systemic Inflammatory Response Syndrome (SIRS) is a common pattern of physiological variables, which occurs following critical injury, severe infection and other physiological insults. It is defined as characteristic combinations of hyper-/hypothermia, tachycardia, tachypnoea and leucocytosis or leucopaenia (Bone et al, 1992). As the name implies, the syndrome arises from immuno-inflammatory activation, which is initiated by networks of cytokines and various other mediators released from tissues during stress. SIRS can range from mild to severe, and may be complicated by sepsis and Multiple Organ Dysfunction (MOD) or Failure (MOF). The main cause of death in ICU patients who initially survive their injury is, in fact, MOF, which is characterised by serial, relentless organ failure (Baue, 1975; Eiseman et al, 1977).

Thus, critical injury and its consequences can be viewed as a powerful stressor. Gut function under these circumstances is of interest for two reasons. Firstly, the gut can be implicated in the aetiological processes, which initiate and/or drive common inflammatory pathways resulting in bacterial translocation, SIRS and MOD (Moore, 1999). Secondly, critically ill patients frequently exhibit features of gastrointestinal dysfunction, such as gastric stasis, ileus and diarrhoea, which to date are largely unexplained. Subsequent sections of this review will expand discussion of these topics, with particular reference to altered gut function in this particular example of stress.

1.2. The gut under stress

1.2.1. Gut motor responses

It has been shown in various animal models, and in humans, that stress (both physical and psychological/emotional) alters gut function. The gastrointestinal tract has a limited portfolio of possible responses to stress (including vomiting, diarrhoea, constipation, altered motility and pain), and these can occur even when the stress is not directed at the gut itself. Almost every study on the subject indicates that physical and/or psychological stress affects gastrointestinal function and produces symptoms in almost everybody, whether patient or healthy volunteer. Even emotions arising from stress exposure are sufficient to induce alterations in gut function in susceptible individuals (Narducci et al, 1985; Welgan et al, 1988).

The different regions of the gastrointestinal tract display characteristic responses to stressful stimuli. This has been demonstrated repeatedly using many different forms of experimental stressor in both animal subjects and studies involving humans. Stress may alter gut motility and transit, or may affect non-motor function as will be outlined below.

A variety of different animal studies have consistently shown that stress delays gastric emptying. Restraint stress has been demonstrated to inhibit stomach emptying in rats (Mönnikes et al, 1992;

Tsukada et al, 2003; Nakade et al, 2005), and inhibition of gastric antrum contractility (Berezina and Ovsyannikov, 2001). This is primarily a psychological stressor, since the animals are not physically harmed in the process of restraint. Similarly, dogs exposed to an unfamiliar environment responded with reduced motor activity of the antrum of the stomach, which impeded gastric emptying (Mistiaen et al, 2002). Impaired gastric emptying has also been shown in animal studies, which utilised various other psychological stressors (Taché et al, 2001). The same is true when the stressful insult is of a physical nature, although clearly a physical insult may well have a psychological overlay in the exposed animal. Examples of stressors which cause delayed gastric emptying in animals are operant avoidance, exposure to radiation, handling, haemorrhage, abdominal or cranial surgery, swimming and anaesthetic exposure (Enck and Holtmann, 1992).

Interestingly, one study reported a different result: - acute cold exposure increased animals' gastric motility in a study by Martinez et al (1998). However, this may not be a completely contradictory finding. A subsequent study has refined our understanding of the mechanism by which the typical stress-induced impairment of gastric emptying may occur. Nakade and co-workers (2006) showed that it might be a result of impaired antro-pyloric co-ordination. This study in rats demonstrated a 34-40% increase in motile activity in the antrum of the stomach and pylorus during restraint stressor application. However, the co-ordinated propagation of contractions through the antrum toward the pylorus (necessary for integration of gastric emptying) was a quarter of that in the unstressed state - a significant difference. Thus, motility changes alone may not explain the gastrointestinal symptoms associated with stress, if contractility becomes uncoupled from the normal functional co-ordination of the gut, as this study suggests may happen. This mechanism adds to the existing evidence for altered motor activity in different gut regions from manometric studies on stressed animals. The work of Berezina and Ovsyannikov (2001) cited earlier, for example, also demonstrated that contractile activity in the distal ileum, ileo-caecal valve region as well as the proximal colon were inhibited, while duodenal contracility was stimulated. This indicates that stress can induce pandysmotility of the gastrointestinal tract, and that regional differences may occur simultaneously.

What do we know about lower gastrointestinal tract responses to stress? Various animal studies, which employed manometry techniques, have investigated this question. Physical exercise in dogs has been shown to induce a defaecation response associated with colonic mass movements and increased duration of colonic contractile activity (Dapoigny and Sarna, 1991). Similarly, increased colonic spike burst activity has been demonstrated in rats exposed to open field test and electrified prods (Stam et al, 1997; Stam et al, 1999). Novelty and noise exposure stress produced a smaller colonic effect, and conditioned fear produced a greatly increased colonic spike burst frequency along with increased defaecation (Stam et al, 1995; Stam et al, 1999). Restraint and cold exposure causes increased faecal output and fluidity, which was shown not to be the result of increased secretory mechanisms or small bowel transit, but due to greatly hastened colonic transit time (Barone et al,

1990; Mönnikes et al, 1992; Martinez et al, 1998). Interestingly, water avoidance stress and tail shock suppressed proximal colon contractility in rats but simultaneously increased defaecation and faecal fluid content (Morrow and Garrick, 1997). Diarrhoea despite suppressed colonic contractility suggests that faecal output may not exclusively involve colonic motor mechanisms. Furthermore, subsequent exposure of the animals to the shock chamber without imposing new shocks also increased faecal output from baseline, suggesting that prior stress can prime future bowel responses to stress.

In general, the small bowel seems more resistant to the effects of stress than other gut regions. Some of the studies discussed above (Barone et al, 1990; Stam et al, 1995) detected no change in small intestinal function – although many studies did not investigate this region of the bowel. Nevertheless, a few studies in rats have shown a generally slowed intestinal transit under conditions of wrap restraint, cold water swim or ether exposure (Taché et al, 2001). This response seems paradoxical given the response of the large intestine. However, regional differences in the enteric response to stress clearly occur.

1.2.2. Gut non-motor responses

Gut non-motor responses are also associated with stress. The gut is a vital physical, chemical and immunological barrier between the internal and external environments. The gastrointestinal tract is composed of four histological layers: the mucosa, the submucosa, the muscularis externa, and the serosa. These few layers of tissue are what separate the external world from the body's internal environment. Mucosal epithelial cells are connected to each other by tight junctions, which function to control the passage of digested nutrients, water, electrolytes and other molecules across this barrier via para- and transcellular routes. At the same time they exclude significant amounts of antigen and toxins. Furthermore, the secretory fluid and mucus production together with immunological products such as IgA provide a defence line against potentially harmful substances including pathogenic agents, such as endotoxin. This ability of the mucosa to control entry of some molecules and bar entry of others is known as the intestinal barrier function. Because these physical and physiological barrier functions of the GIT are under neurohormonal influence, they too can be impacted by stress.

In rodents, experimental stress appears to disrupt the gut epithelial barrier. Restraint stress results in increased intestinal ion secretion and increased permeability of both the small intestine and colon (Saunders et al, 1994; Kiliaan et al, 1998; Santos et al, 2000; Santos et al, 2001; Cameron and Perdue, 2005). The increased permeability affects both large nutrients and potentially harmful materials such as antigens and pro-inflammatory substances that should normally be excluded from the body. Furthermore, there is evidence that normal transport mechanisms for valuable nutrients - sodium coupled glucose absorption, for example - may be unfavourably affected as well (Boudry et al, 2007). Disruptions of the epithelium as well as the endothelium have been shown in stressed animals. These structural disruptions have deleterious effects on the cellular exchange barrier in the tissue

(Wilson and Baldwin, 1999). This effect may not be solely acute, since the handling and transport of animals has been shown to disrupt intestinal barrier function for up to 2 weeks (Meddings and Swain, 2000).

Stressors have been shown to increase intestinal fluid secretion and colonic mucin production in a series of animal studies (Empey and Fedorak, 1989; Castagliuolo et al, 1996a; Castagliuolo et al, 1996b; Castagliuolo et al, 1998). This may be an important compensatory mechanism for protecting the internal environment from invasion in the face of increased permeability. In a related finding, Spitz et al (1996) demonstrated that mimicking stress via glucocorticoid administration caused increased bacterial adherence to the mucosa, and that this effect is associated with reduced IgA and increased intestinal permeability. Thus, the effects of stress seem to increase the vulnerability of the organism to invasion, by altering the manner in which luminal bacteria are able to interact with the mucosa. This, together with findings that the microflora populations may be unfavourably altered (i.e. a reduction in the ratio of commensals) in stress, highlights the importance of appropriate gut flora populations as part of mucosal barrier defences (Bailey and Coe, 1999).

1.2.3. Gut responses in man

The above paragraphs have summarised the known general effects of stress on the gut from available animal data. Due to obvious ethical considerations similar demonstrations in human subjects can be challenging. Experimental stressors are more difficult to design and human gut tissue for in vitro study can be difficult to procure, not least from healthy persons. On the other hand stress *perception* and the influence of the emotions (limbic input) - both components of the psychological nature of stress - can be investigated readily in man. In fact, in human beings, strikingly similar stress-induced effects on the different gut regions have been incidentally or purposefully shown.

Persons participating in hard running exercise frequently report loose stool, nausea, vomiting, faecal urgency, abdominal cramps, faecal incontinence and even rectal bleeding during or immediately following exercise activity (Moses, 1990; Sullivan and Wong, 1992; Butcher, 1993). Gastric emptying in the exercising individual depends on multiple factors such as intake volume, calorie content, osmolality, and temperature. Nevertheless, bowel symptoms in athletes are so frequent and so unrelated to demographic indicators that it supports an exercise-effect as an explanation. In addition to exercise, other physical stressors, such as irradiation (Yeoh et al, 1993), general anaesthetic (Schurizek, 1991), labyrinthine stimulation (Muth et al, 1996) and short-term starvation (4 days) (Corvilain et al, 1995) also retard gastric emptying in man.

Psychological stress results in variable effects on gastric motor activity and emptying. Pre-operative anxiety slows gastric emptying, dichotomous listening slows gastric emptying in some individuals, and anger induction seems to reduce gastric emptying in patients with Irritable Bowel Syndrome (IBS) but

not normal controls (Magni et al, 1991; Welgan et al, 2000; Taché et al, 2001). In fact, patients with IBS, as well as those with organic disease such as Inflammatory Bowel Disease (IBD) commonly report that life stress is associated with greater bowel symptoms (Whitehead et al, 1992; Mawdsley and Rampton, 2005).

Psychological stress seems to affect colonic function in particular. Mirror drawing, an artificial mental stressor, increased colonic motility in human subjects, as did a stressful interview situation (Almy et al, 1949; Fukudo et al, 1987). Dichotomous listening has been shown to heighten rectal and anal perception and enhance colonic motor activity (Rao et al, 1998; Murray et al, 2004). Anger induced by criticism of intelligence test performance or delay in providing assistance for a routine diagnostic procedure significantly augmented colonic motor and spike activity (Welgan et al, 1988). The Stroop test and ball sorting stress (Narducci et al, 1985) also increased colonic motility, but only on the first (and not repeated) exposure, suggesting that the novelty of the stressor may contribute to the response.

Physical stressors such as ice water immersion of the hand or foot also increase anorectal perception and stimulate colonic motor activity and motility (Narducci et al, 1985; Rao et al, 1998; Murray et al, 2004). Some data from these studies suggests that this response is potentiated when a psychological stressor is superimposed onto cold pressor stress. In fact, it is extremely difficult, both in experimental conditions and in real life, to separate psychological or emotional components under conditions of physical stress.

Various stressors have also been shown to affect non-motor gastrointestinal functions in man, just as in animals. Dichotomous listening and moderate exercise reduced net intestinal water, sodium and chloride absorption and resulted in a secretory profile in the human jejunum (Barclay and Turnberg, 1987; Barclay and Turnberg, 1988a). The same group and others later demonstrated the same effect as a result of cold pain stress (Barclay and Turnberg, 1988b; Santos et al, 1998). What is the clinical relevance of such findings? Stress-induced gastrointestinal symptoms may be difficult to identify as originating in the small intestine. However, the increase in volume of secreted fluids in the small bowel together with accelerated colonic transit may account for diarrhoea, which is commonly recognised to accompany stress. The implication of altered ion and fluid secretion is that intestinal transport function, and therefore barrier integrity, is affected. This has been shown to be true for physical stressors such as abdominal surgery (Jiang et al, 2003; Matejovic et al, 2004), aortic aneurysm repair (Lau et al, 2000), and open cholecystectomy (Schietroma et al, 2006). All of these procedures and many other clinical conditions, such as shock and sepsis, have been associated with increased intestinal permeability (Ziegler et al, 1988; Deitch et al, 1996). Information that is largely absent in man is the effect of purely psychological stress.

Taken together, the evidence indicates that the motor response to stress is generally one of inhibition in the upper gastrointestinal tract and stimulation in the lower gastrointestinal tract, with the colon being more responsive to stress than the rest of the gut. The small intestine seems to be affected mainly in a manner in which gut barrier integrity and function is altered. Broadly, the responses of the different bowel regions to stressors are remarkably unchanged with changing stressors. The mechanisms by which these responses occur are not yet entirely clear, but existing data will be discussed in subsequent sections.

1.2.4. Gut function in the critically ill

A functional gut in the sick patient is of considerable importance and clinical relevance. Nutritional support is considered standard care for the critically ill. Current recommendations are that the enteral route is the preferred method of nutrition delivery. This is because at least some important clinical outcomes (such as infectious complications) are improved through the use of enteral nutrition (Gramlich et al, 2004). However, impaired gastric emptying, disordered motility and diarrhoea are commonly observed in critically ill patients (Ott et al, 1991; Heyland et al, 1996; Montejo, 1999; Ritz et al, 2000; Chapman et al, 2004; Chapman et al, 2005; Nguyen et al, 2007; Landzinski et al, 2008). Reportedly at least half of patients on mechanical ventilation and up to 80% of head-injured patients are affected by retarded stomach emptying (Tarling et al, 1997; Kao, 1998). Patients with neuro-trauma in fact have among the highest occurrence of disturbed gastric emptying of all ICU patients, along with those with multiple trauma, burns and sepsis (Nguyen et al, 2007). Such alterations in gut function are associated with uncomfortable symptoms for the patient (including nausea, vomiting and abdominal distension), and significantly hamper provision of nutritional requirements. Additionally, potentially dangerous complications such as aspiration pneumonia can result from gastric stasis and reflux of even low volume contents of a non-motile stomach into the airway.

Although intensive care medicine has advanced rapidly over the past two decades, surprisingly little attention has been paid to the investigation of gut dysfunction in the ICU. For such an important and well-known phenomenon, good prevalence data on bowel dysfunction in this population is scarce. Certainly it is a difficult matter to study: - multiple confounding factors related to clinical condition, medical and surgical interventions and a host of other iatrogenic influences have an impact on the gut. The widespread use of opioids may be foremost among these (Moniche et al, 1995; Morimoto et al, 1995; Kehlet and Moesgaard, 1996; Steinbrook, 1998; Liu and Wu, 2007). Other factors such as the use of sedation, analgesics, inotropic support, and surgery involving manual manipulation of intestines by the surgeon's hands, logically must impact on gut motor function. All of these factors are well known and are virtually inevitable causes of gastric motor stasis in the post-operative period (Kehlet, 1997; Mattei and Rombeau, 2006). At least some modes of mechanical ventilation (such as positive pressure) may cause poor splanchnic perfusion thus also leading to gastrointestinal complications (Mutlu et al, 2003). Further, motility disturbances are a known complication of many

clinical conditions including MOF, an unstable haemodynamic state, and raised intra-abdominal pressure.

So what is known of bowel abnormalities in the ICU setting? Once again, differences between the various gut regions seem to exist. Two small studies have looked at oesophageal function in the critically ill. Using 24-hour manometry in 15 mechanically ventilated patients, Kölbel et al (2000) showed markedly inhibited oesophageal motility patterns in patients on any of various analgesic and sedative medications. A further study into oesophageal function in 15 similar patients demonstrated drastically reduced lower oesophageal sphincter pressure and poor oesophageal peristaltic activity, which allowed frequent gastro-oesophageal reflux to occur (Nind et al, 2005).

Feed intolerance related to gastric stasis is perhaps the most common reason for failure of enteral feeding in ICUs. A series of manometric studies in mechanically ventilated patients using healthy volunteer control subjects, showed abnormalities in the characteristics of motility patterns in the upper gastrointestinal tract. In the first, the stomach was shown to exhibit marked reductions in antral contractile activity with a complete absence of the high-frequency contractions associated with the propulsive phase of the inter-digestive motility (fasting state migrating motor complex) pattern (Dive et al, 1994b). This result was confirmed by Bosscha et al (1998) who demonstrated significantly shorter periods of stomach contractile activity together with poor gastric emptying in mechanically ventilated patients. Both of these studies also demonstrated abnormal inter-digestive motility that disrupts the full expulsion of gastric contents that should normally occur, and therefore causes gastric retention. Further, in some patients, the contractile motor waves did not propagate correctly. They remained stationary or moved in an oralward instead of analward direction, and provoked reflux from the duodenum in a retrograde fashion into the stomach (Dive et al, 1999).

Bosscha's results (1998) and those of a related study by Dive's group (1994a) also indicated that feeding of critically ill patients did not interrupt the inter-digestive or fasting state motor pattern of the duodenum as should normally occur. More recently, an investigation of pyloric motor activity in the critically ill has supported these reported patterns of persistent but unusual duodenal motor activity. Feeding was shown to suppress gastric contractility but stimulate pyloric tone, an effect not seen in healthy persons (Chapman et al, 2005). This response was once again associated with significantly delayed gastric emptying. Thus, among this patient population there is both a basal and a responsive gastrointestinal motor abnormality, at least in the upper gut.

Apart from what has been discussed above on duodenal responses, virtually no information exists regarding the response of the rest of the small bowel in critical illness. Manometry beyond the duodenum is technically exceptionally challenging in the human subject. However, one study used a 200cm multi-channel system to record intra-luminal pressures simultaneously in the gastric antrum,

duodenum and proximal jejunum of patients undergoing elective repair of infra-renal aortic aneurysm (Tournadre et al, 2001). The manometry catheter was placed in the operating theatre and data was collected over 1-5 days in 11 patients. The results indicated bursts of motor activity in the duodenum and proximal jejunum (but not the stomach) within only 2 hours of this major surgical procedure, which involved significant handling and mobilisation of the intestines. As with earlier studies, the nature of the motor activity was unusual, sometimes involved deranged direction of migration, and did not change to a fed state pattern once patients were enterally fed. This is particularly interesting data since it includes information from up to 5 days post-operatively whereas previous studies have been of very short duration. It suggests two things: firstly, that the small bowel is not inhibited in the same way as the stomach is, and secondly, that there seems to be one common small intestinal response, which manifests as active but abnormal motility.

Symptoms that are interpreted to be colonic in origin i.e. changes in stool frequency, form and consistency are common in the ICU. Constipation is seen with equal frequency as other common gastrointestinal symptoms (Montejo, 1999). This is understood to be related to the well known post-operative slowing of colonic transit seen in a wide variety of patients (Wilson, 1975; Muller et al, 1985; Tolleson et al, 1992). Of course many drugs are inhibitory on colonic motility too, and may have an additive effect in prolonging colonic transit. Conversely, diarrhoea is very widespread in the ICU. It may be experienced by up to 50% of patients during an ICU stay (Kelly et al, 1983; Ringel et al, 1995). Myriad iatrogenic and clinical factors probably combine to induce this symptom though. Not least of these many things are the hypo-albuminaemia associated with severe illness, and the extensive use of antibiotics (Mutlu et al, 2001). Furthermore, the findings mentioned in preceding paragraphs indicate that diarrhoea may not necessarily be due to a colonic problem. This evidence suggests that small bowel motor activity is persistent and atypical in the critically ill. Because such motility is propulsive in nature, it may in fact be at least part of the cause for apparently idiopathic diarrhoea in the ICU patient, as intestinal contents may move more rapidly through the gut.

Poorly synchronised gastrointestinal motor activity in very ill patients also raises another concern. If, as data suggests, motility disturbances impair the clearance and elimination of intestinal contents, the critically ill patient may be at risk of prolonged exposure to antigenic and microbiological agents, which remain in contact with the mucosal surface. The intestinal barrier is compromised in the critically ill, whether or not their illness/injury involves the gastrointestinal tract. Burn injury as well as polytrauma is associated with enhanced intestinal permeability, especially when accompanied by circulatory shock (Deitch, 1990; Ziegler et al, 1988; Doig et al, 1998; Faries et al, 1998; Magnotti and Dietch, 2005).

Shock might cause mucosal hyper-permeability in a number of ways. Firstly, the metabolic demands of the gut tissue, which incorporates epithelial, molecular and immune barrier components, may not

be met under conditions of poor gut perfusion. Nitric oxide-dependent as well as acidosis- or hypoxia-induced cell injury and apoptotic villous loss may occur (Rombeau and Takala, 1997; Holland et al, 2005; Rupani et al, 2007). This could have critical knock-on effects on protein expression and molecular transport that in turn impact on mucosal integrity. For example, it has been shown in animals that severe injury results in a marked reduction in intestinal levels of membrane-associated proteins (such as occludin and zona occludens protein-1), which are crucial regulators of intestinal tight junctions (Han et al, 2002; Yang et al, 2003; Constantini et al, 2008). Similarly, shock-associated disruption of the mucosal mucus layer and changes in mucus qualities have also been directly linked to enhanced intestinal permeability in a small animal model (Rupani et al, 2007).

Secondly, inflammatory cytokine responses in shock are inextricably linked to enhanced gut permeability (McKay and Baird, 1999). As a mechanism for inducing gut permeability this makes sense because injuries distant from the gut result in similarly impaired gut permeability as direct gut insult does. Many published reports demonstrate that shock and trauma are associated with increased pro-inflammatory cytokine expression, both in the gut and in distant tissues (Roumen et al, 1993; Martin et al, 1997; Seekamp et al, 1998; Ayala et al, 1999; Meng et al, 2001; Yang et al, 2002a; Yang et al, 2002b; Yang et al, 2003; Fink, 2003). Furthermore, the increase in these cytokines, such as interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α), are fundamental to the development of intestinal hyper- permeability (Yang et al, 2003; Diebel et al, 2005; Spindler-Vesel et al, 2006).

Ischaemia-reperfusion injury to the gastrointestinal tract may be a third mechanism whereby shock causes intestinal hyper-permeability. This is particularly plausible in the gut because shock results in diversion of blood to other more critical organs as an essential strategy to favour survival. Besides the resultant local production of free radicals and cytokines that occurs in low flow events, as has been discussed above, reperfusion itself may be damaging to the gut. Loss of intestinal barrier function is associated with epithelial cell apoptosis, which seems to be correlated with the severity of the reperfusion injury (Shah et al, 1997; Noda et al, 1998; Sun et al, 1998; Hotchkiss et al, 1999; Hotchkiss et al, 2000; Ikeda et al, 2002; Zhang et al, 2002). Chang et al (2005) showed that morphological changes are more pronounced in the small intestine than in the colon following an ischaemia-reperfusion episode. These structural changes included villous cell swelling and degradation as well as substantial denuding of the villi themselves, as has also been shown in more recent work (Higuchi et al, 2008). Both apoptosis and necrosis of cells, as well as opening of tight junctions and reduced mucosal thickness were all demonstrated in Chang's (2005) experiment. This structural damage was correlated with a massive increase in intestinal permeability, with the most severe destruction of cells being associated with the time of resuscitation following ischaemia. However, restitution of the mucosal did not immediately reverse the increased permeability indicating that functional changes in the gut barrier are not necessarily a result only of morphological impairments.

As with shock, conditions such as sepsis, severe acute pancreatitis, and inflammatory bowel disease are associated with a generalised hyper-permeability of the small intestine, demonstrated by enhanced absorption of a probe molecule via an intestinal paracellular route. (Lara and Jacobs, 1998; Jiang et al, 2003; Matejovic et al, 2004). It has recently been shown, however, that colorectal permeability is also significantly increased in patients with severe sepsis and septic shock compared with healthy controls (Jørgensen et al, 2006). Although there were some limitations to this study, it is the first such finding in the critically ill. It is an important one, because the colon contains enormous bacterial populations. Mucosal permeability under these conditions may have adverse consequences for the stressed patient.

Enhanced intestinal permeability has been suggested to permit the translocation of bacteria and/or endotoxin from the gut lumen, where they are normally resident, into the body. This is known as bacterial translocation. Endotoxaemia has been long shown in shocked trauma patients and following major surgery, and is correlated with intestinal permeability (Rush et al, 1988; Moore et al, 1992; Lau et al, 2000). Although there have been suggestions of translocation of enteric organisms to remote sites in the body (MacFie et al, 1999), there has never been completely convincing human evidence for the translocation of bacteria through a permeable gut. Thus, the once popular (and not yet completely discredited) theory of the "gut hypothesis of MOF", which proposed a link between bacterial translocation, SIRS, increased systemic infections and sepsis-associated MOF in ICU patients is no longer in the forefront (Moore, 1999; Deitch, 2002; Alverdy et al, 2003; Deitch et al, 2006; Senthil et al, 2006; Reddy et al, 2007; Alverdy et al, 2008). Instead, interest is currently focussed on the role of micro-aspiration of bacteria from a hypo-motile, colonised stomach into the airway, causing infectious complications such as pneumonia (Gatt et al, 2007). This alternate focus also implicates the gut in sepsis-associated organ dysfunction, but not via the originally proposed mechanism of translocation.

The precise mechanisms of altered gut function in the critically ill are as yet unclear. It probably results from a combination of factors which disfavour good bowel function. Apart from those already mentioned, such factors may include the historically enforced supine position, the use of opioids, antibiotics and other medications which impact on gut function, together with underlying physiological and structural changes in the gastrointestinal tract. It is a matter of clinical import, however, since bowel dysfunction is associated with increased morbidity and poor outcome, including death, in critically ill patients (Moore et al, 1992; Ziegler et al, 1994; Montejo, 1999; Ritz et al, 2000).

1.3. The role of corticotropin-releasing factor in stress-related gut dysfunction

While the effects of stress on the gut are well described, the mechanisms underlying these effects have not been entirely elucidated. The brain-gut axis has become increasingly recognised as important in gastrointestinal disorders and diseases, rather than just a means of regulating normal gut

functions. This has generated a massive research interest in the topic of brain-gut interactions, and over the past decade or two this area of study has been accelerating. What has really allowed great progress in this field is the understanding that it is a subject of research that requires convergence of many different disciplines. The interface of expertise in physiology, gastroenterology, neurobiology and immunology, for example, has been essential in providing what information we have to date.

The identification of brain peptides that impact upon the function of the gastrointestinal system has unlocked our understanding of how the brain-gut axis operates. Demonstration of the functions of these molecules at central nervous system, spinal cord and enteric nervous system levels has been crucial in advancing knowledge of the inter-connectedness of the gut with the brain. One such brain peptide is corticotropin-releasing factor (CRF). It has become apparent that the stress effects on the gut are mediated via CRF. In fact, evidence has been accumulating which convincingly indicates that CRF is *central* to the brain-gut response to stress.

As has been mentioned, CRF released during stress activates the HPA axis, and this endocrine function has somewhat dominated the study of CRF since its discovery. However, it is now clear that this molecule is also able to exert endocrine and motility effects quite independently of the HPA axis. This is extremely important and is a critical point to understand: – while other releasing factors/hormones only produce effects if they act in the brain, CRF acting in the periphery has effects of physiological importance. Furthermore, the development of biological agents that act as CRF receptor antagonists have contributed greatly to clarifying our knowledge of the physiological relevance of CRF, as it has allowed the demonstration of the receptor-mediated action of CRF.

CRF is a 41-amino acid peptide of the peptide family that includes CRF (Vale et al, 1981) and other related mammalian peptides. These are urocortin (Vaughan et al, 1995), urocortin II (also called stresscopin-related peptide) and urocortin III (also called stresscopin) (Lovejoy and Balment, 1999; Hsu and Hsueh, 2001; Lewis et al, 2001; Reyes et al, 2001). Other homologous peptides are fish urotensin I (Lederis et al, 1982) and frog sauvagine (Montecucchi and Henschen, 1981). CRF is abundantly expressed in neurons of the paraventricular nucleus of the hypothalamus. These neurons stimulate ACTH secretion, and thereby the pituitary-adrenal axis. The CRF peptide, however, is also expressed throughout the brain of mammals in areas including the hypothalamus, parts of the amygdala, geniculate nucleus, Barrington's nucleus, olfactory bulb, inferior olive and dorsal motor complex (Merchenthaler et al, 1982; Turnbull and Rivier, 1997). This widespread distribution may indicate that there are a variety of functions of the molecule. In summary, it is known to be anxiogenic and to induce alertness. It is anorexogenic and induces brown fat thermogenesis, and therefore has a role in energy balance and metabolism. Additionally, CRF stimulates the cardiovascular system and has regulatory functions in reproduction (Asakura et al, 1997; DiBlasio et al, 1997; Simoncini et al, 1999; Richard et al, 2002; Hillhouse and Grammatopoulos, 2006). CRF is also synthesised and

released in multiple peripheral tissues including skin (Lytinas et al, 2003), gut (Kawahito et al, 1994; Kawahito et al, 1995; van Tol et al, 1996; Muromatsu et al, 2000; La Fleur et al, 2005; Porcher et al, 2006), thymus, spleen, heart, spinal cord, adrenal gland (Thompson et al, 1987), inflammatory sites (Crofford et al, 1993; Mastorakos et al, 1995; Baigent et al, 2000; Uzuki et al, 2001) and immune cells (Stephanou et al, 1990; Ekman et al, 1993).

In the brain, CRF production and release is governed by feedback systems incorporating glucocorticoids, CRF itself, cytokines, and other substances such as lipopolysaccharide (Lightman et al, 1993; Mastorakos et al, 1993; Takemura et al, 1997; Itoi et al, 1998; Grinevich et al, 2001; Yao et al, 2008). Controls such as these are in place so as to precisely regulate the amount of HPA axis end-products circulating in plasma, and therefore regulate allostatic adaptation in the stressed individual. It is critical to limit excessive CRF activity to prevent potentially dysfunctional disturbances in immunity and metabolism (McEwen, 1998; Schulkin et al, 1998).

In mammals CRF exerts its actions through two known G-protein-coupled receptors, namely CRF type 1 (Perrin et al, 1993), and CRF receptor type 2 (Lovenberg et al, 1995a). These receptors are encoded by two different genes, on chromosomes 17 and 7 respectively (Hillhouse and Grammatopoulos, 2006). CRF1-receptor is found in the brain cortex, hypothalamus, locus coeruleus, limbic region, cerebellum and pituitary (Potter et al, 1994). Multiple splice variants of the CRF1 receptor have been identified, but CRF1α is the main functional receptor variant, for which CRF has particularly high affinity. The other receptor variants identified to date have not been shown to have any physiological function, and in fact have critical mutations or deletions, which impair ligand binding and signalling. However, the expression of the different variants is fairly specific to different tissues. It is possible that alterations in the ratio of expression of CRF1α to less functional variants serves to modulate tissue responses to CRF and its related peptides. Of particular interest in humans is that the CRF1 receptor is expressed in a large number of peripheral tissues including the reproductive organs, adrenals, dermal tissue and adipose, heart and the spleen as well as various immune cells (Asakura et al, 1997; DiBlasio et al, 1997; Chatzaki et al, 2004a; Chatzaki et al, 2004b). This does not seem to be the case in other animals, and possibly suggests refinements in the roles of the CRF-like molecules in human physiology (Hillhouse and Grammatopoulos, 2006).

Three functional variants of CRF2-receptor have been identified: - originally designated CRF2α, 2β and 2γ, they are now known as 2a, 2b, and 2c (Taché and Bonaz, 2007). These splice variants differ only in the number of amino acids in their amino terminal extra-cellular domains, the region involved in binding. All mammals appear to express both CRF2a and CRF2b, albeit in different tissues in a species-specific manner. Both variants are expressed in the brain of mammals. However, type 2a is predominantly a brain neural receptor in rodents, whereas type 2b is widespread in non-neural brain tissue and primarily expressed in various peripheral tissues (Lovenberg et al, 1995b). By contrast, in

human peripheral tissue (as well as sub-cortical brain tissue) the dominant receptor type is CRF2a, notably in the human gastrointestinal tract (Muromatsu et al, 2000). The final variant – CRF2c – has thus far been identified only in the human brain, although it may be present in other primate species (Kostich et al, 1998). CRF binding to CRF2 receptors is at much lower affinity than to CRF1 receptors. The urocortins, however, preferentially signal via CRF2 receptors (Taché and Bonaz, 2007).

The development and use of CRF receptor antagonists has been a powerful research tool in clarifying the roles of the receptors in the integrated stress response. In their early designs, antagonists were not specific for the different receptor subtypes and therefore the differential roles of the various CRF receptor variants could not be verified. However, more recently, competitive peptide antagonists have been developed, which bind selectively to the CRF2 type receptor functional variants with equal affinity, but not to CRF1-receptors (Rivier et al, 2002). There are still no useful peptide analogs selective only for CRF1-receptor, although small molecule non-peptide antagonists selective for CRF1-receptor are now available (Chen, 2006). The scientific use of these antagonists has revealed differences in the biological effects of CRF when it interacts via distinct receptor types. In broad terms, CRF1-receptor mediated effects seem primarily related to the HPA axis and sympathetic nervous system driven stress adaptation. This includes anxiogenesis, cardiovascular and immunological functions and effects on the large bowel (Taché and Bonaz, 2007). Interactions with CRF2-receptor types seem to result in regulation of the HPA by maintaining the drive of the HPA axis, and by facilitating recovery of the axis-driven response (Coste et al, 2000). Thus, the impact of the CRF2-receptor ligand binding seems subtler. Stress effects on gastric motility also appear to be associated with CRF type 2 receptor binding, without affecting colonic function (Martinez et al, 2002).

Apart from receptor binding, CRF also binds to CRF-binding protein with high affinity, as does urocortin (Ardati et al, 1998). In rodents, this 37-kilodalton protein is expressed only in the brain where it is associated with cell membranes (Baigent et al, 2000). In humans, however, it is produced in the liver and placenta, circulates in blood and is also found in a number of brain regions including the cortex and hypothalamus (Linton et al, 1988; Potter et al, 1991; Potter et al, 1992; Linton et al, 1993; Behan et al, 1995). Indeed 40-60% of brain CRF is bound by CRF-binding protein (Jahn et al, 2002). This binding protein seems to be a pseudo-receptor, acting as a means by which the endogenously produced CRF peptide can be made differentially available to target tissues. Since it is highly co-localised with CRF and CRF receptor distribution it may function to modulate the availability of CRF to its receptors, and therefore limit its action (Potter et al, 1994). Binding affinity of the CRF binding protein, however, does seem to differ from that of the CRF receptor. The presence of CRF binding protein in the brain, for example, does not block the response of the pituitary to hypothalamic CRF, yet circulating CRF binding protein rapidly clears CRF from the plasma (Linton et al, 1990; Orth, 1992). The exact contribution of the CRF-binding protein to CRF mediated effects is still under investigation.

The above paragraphs have given context as to the characteristics of the CRF peptide, its ligands and receptors. General comments have also been made regarding the known functions of the CRF signalling system. Since the stress response of the gastrointestinal tract is the focus of this review, the sections that follow will review more precisely and in more detail how CRF is understood to impact on this organ system.

1.3.1. CRF and gastrointestinal motility

To recap, the motor effects of stress on the gut are generally recognised to be inhibition of the upper gut and stimulation of the colon. Animal studies have shown that the injection of CRF into the cerebrospinal fluid mimics the inhibition of gastric motility induced by stress (Bueno and Fioramonti, 1986; Garrick et al, 1987; Lee and Sarna, 1997). This indicates that delayed gastric emptying is mediated by centrally acting CRF. The gastric transit of solid, liquid and acaloric liquid meals are all affected. This is now known to be a receptor-mediated action, mainly via the CRF2 receptor sub-variant, since injection of CRF2-receptor antagonists into cerebrospinal fluid completely prevents the impairment of gastric emptying associated with a range of stressors (Taché et al, 1999). The specific site of action is the paraventricular nucleus (PVN) of the hypothalamus and the dorsal vagal complex, which both contain CRF2a expressing neurons (Martinez et al, 1998; Taché et al, 1999). This data converges with the demonstration that a number of physical stressors activate CRF neurons and cause upregulation of CRF mRNA in the PVN (Harbuz and Lightman, 1989; Rivest and Rivier, 1994). This hypothalamic CRF effect is not via the HPA, however, since surgical removal of the pituitary or adrenals does not change the gastric inhibitory effect of central CRF (Taché et al, 1987; Lenz et al, 1988).

The peripheral (intravenous or intraperitoneal) injection of CRF also inhibits gastric emptying in various mammals (Webster et al, 1996; Nozu et al, 1999). This occurs because intravenous CRF reduces the amplitude of digestive pattern gastric contractile motor activity and inhibits jejunal motility (Bueno and Fioramonti, 1986). Peripheral CRF does not appear to cross the blood-brain barrier, so the effect does seem to be locally mediated. Furthermore such effects can be replicated in isolated gastric preparations, which have functional enteric neurons – this supports a localised action (Porcher et al, 2006). In this experiment, exogenous CRF was applied to mounted strips of viable rat gastric antral muscle, which had been stripped of the mucosa. CRF had the effect of reducing the amplitude of contractile activity compared with the basal levels of spontaneous active contractions which occurred in the muscle isolates. The inhibitory effect of CRF was abolished by the simultaneous application of a neurotoxin, indicating that the CRF-induced inhibition in antral motility occurred via enteric nerves.

While the gastric inhibitory effect of stress can be replicated via either central or peripheral CRF injection, evidence reveals that the site of action is different. For instance, pharmacological autonomic

nervous blockade does not change the delay in gastric emptying that results from peripheral injection of CRF. However, it does prevent the gastric inhibition produced by cerebrospinal CRF injection (Lenz et al, 1988). Data from other animal studies indicates that the gastric effect of peripheral CRF may be via CRF2-receptors, because peripheral administration of specific CRF2-receptor antagonists prevented the slowed gastric emptying that results from peripheral (intravenous or intraperitoneal) CRF injection (Martinez et al, 2002; Million et al, 2002). Stress-induced gastric stasis is also prevented by peripheral administration of CRF2-receptor antagonists to animals under stress such as restraint and abdominal surgery (Taché et al, 1991; Martinez et al, 1998). In contrast, gastric stasis following a surgical stress in mice has been shown to be via CRF1-receptors, with no effect by CRF2-receptor antagonists (Luckey et al, 2003). The stress of abdominal surgery is known to activate CRF pathways and these findings suggest that peripheral CRF receptors might be important in post-operative ileus (Taché et al, 1991). Interestingly, CRF antagonists administered centrally are also effective in preventing post-operative ileus suggesting that both central and peripheral CRF mechanisms are specifically involved in surgery-induced gastrointestinal dysfunction (Taché et al, 1999).

The colonic motor response to stress (increased motility, accelerated transit) can be mimicked by injection of CRF into the cerebrospinal fluid or the PVN of the hypothalamus (Williams et al, 1987; Mönnikes et al, 1993). This response can be blocked by centrally administered CRF receptor antagonists (Saito et al, 2005). It is also true that central administration of CRF antagonists blocks the colonic dysfunction associated with various stressors in rodents (Martinez and Taché, 2001). Basal colonic function, however, is unchanged by CRF antagonists given to unstressed animals suggesting that endogenous CRF is required for the colonic response (Santos et al, 1999). It has been shown the colonic stress response is mediated via CRF1-receptors, since central selective CRF1 receptor blockade prevents it (Martinez and Taché, 2001). Neither do the CRF2 receptor blockers that prevent gastric retardation have an impact on colonic stimulatory activity (Martinez et al, 2004). Finally, CRF1-receptor null mice have overall lower defaecation scores when exposed to stressors, compared with wild-type animals (Bale et al, 2002).

CRF delivered into the peritoneum results in colonic spike-burst activity and defaecation response (Martinez et al, 2002; Miampamba et al, 2002). This occurs with a similar potency as with central CRF effects on the colon. The effect of intravenous CRF is also to accelerate colonic transport. The mechanism of action is not via brain CRF receptors, since central use of CRF receptor antagonists cannot prevent the effect (Lenz et al, 1988). Rather the colonic motility effect seems to be mediated via colonic CRF1-receptors. This has been demonstrated in animal studies, which showed firstly that peripheral administration of selective CRF2-receptor antagonists did not influence colonic transit. Secondly, peripheral injection of selective CRF1-receptor antagonists blocked the colonic motor activity and defaecation induced by peripheral CRF delivery (Martinez et al, 2002). Thirdly, the stress-

related stimulation of colonic activity could be blocked by CRF1-receptor antagonists given peripherally (Maillot et al, 2000).

Thus, to summarise animal data, CRF is able to mimic the manner in which stress alters gut function. This occurs via both central and peripheral mechanisms. The mechanisms involved include a number of receptors for CRF and related peptides, but do not rely on an intact HPA. In general, the CRF2-receptor appears to be mainly responsible for gastric and upper gut stress effects. In contrast, colonic responses seem governed mainly by CRF1-receptor binding.

The importance of CRF in the gastrointestinal response to stress in humans is far from well defined, although it seems probable that it has a major role. An early publication showed that intravenous CRF injection increased motor activity in the duodenum (Mayer et al, 1992). Subsequently, Fukudo et al (1998) administered an intravenous bolus of CRF (a dose that raised ACTH to levels seen in stressed individuals, with measurable plasma CRF) to human subjects while performing continuous colonic and duodenal manometry. Within minutes of CRF injection, duodenal and colonic motility patterns were significantly increased from baseline. This was more pronounced in subjects with IBS, but also occured in healthy volunteers. This study in humans confirmed the impact of exogenous CRF on intestinal contractility, and virtually no similar *in vivo* human data has been published since. The same group (Sagami et al, 2004) more recently showed that peripheral administration of a non-selective CRF antagonist significantly reduced anxiety as well as exaggerated colonic motility responses to rectal stimulation in IBS subjects. In this study tolerance to rectal distension was significantly increased in normal controls. Both of these effects occurred without suppression of the HPA axis. Thus, there is evidence that stress impacts on the release of CRF (or its ligands) in the gut from where it can locally effect changes in gastrointestinal motility.

1.3.2. CRF and intestinal barrier function

As with gut motor changes, stress-induced barrier dysfunction such as increased ion secretion and intestinal permeability can also be replicated by peripheral injection of CRF and prevented by CRF antagonists in animal models (Santos et al, 1999; Saunders et al, 2002b). Similarly, injection of CRF into unstressed animals enhanced colonic mucus secretion, ion secretion, watery diarrhoea and macromolecule permeability (Castagliuolo et al, 1996a; Saunders et al, 2002a; Saunders et al, 2002b). The response was abolished when the animals were pre-treated with a CRF antagonist. Saunders et al (2002b) further showed in vitro using Ussing chamber mounted tissue specimens of full thickness viable rat bowel that addition of CRF to the fluid bath massively increased lumen-to-serosa flux of large molecules and greatly increased mucosal ion secretion. Moreover, the CRF-mediated stress-induced barrier dysfunction was unaltered by blocking adrenal steroids in intact animals – therefore, adrenal glucocorticoids may not be involved in the mechanism. Similar data has recently emerged from an experiment using colonic biopsy specimens from healthy volunteers

(Wallon et al, 2008). In this study, exposure of biopsies to exogenous CRF caused increased mucosal permeation of large molecules. This effect was blocked when CRF-receptor blockers were applied.

Therefore, CRF receptors located peripherally appear crucial for the intestinal epithelial functional impairment induced by stress. At first this was thought to be mediated mainly by CRF1-receptors. This comes from data that stressed rats have increased colonic epithelial permeability as a result of stress, and that this is prevented by the administration of an antagonist selective for the CRF1-receptor (Barreau et al, 2007). Wallon's study (2008), however, demonstrated that the CRF-induced permeation effect could be partially blocked by selective antagonists for both CRF1- or CRF2-receptor, and completely blocked by a non-specific CRF receptor antagonist. Recently Teitelbaum et al (2008) also showed that both CRF-1 and CRF-2 are implicated in barrier dysfunction, but in different ways. CRF-1 is associated with a hyper-secretory state, while CRF-2 is associated with hyper-permeability.

1.3.3. CRF and neural circuits

The exact neural circuitries whereby CRF can interact with CRF1 and CRF2 receptors are not entirely clear. It is probable that peripheral CRF does not cross the blood-brain barrier to act centrally. Rather circulating CRF is more likely to impinge on CRF neurons in the circumventricular organs (area postrema, median eminence), which are outside the protection of the blood-brain barrier. Specifically, central CRF-mediated gastric emptying impairment appears quite independent of the HPA axis, and relates to altered Autonomic Nervous System (ANS) activity, certainly including the vagus (Chen et al, 2002; Czimmer et al, 2006; Tsukamoto et al, 2006;). Activation of the ANS allows stress effects to be rapidly manifested in the gastrointestinal tract. This is because the peripheral autonomic nervous system interacts with the enteric one via sympathetic, vagal and parasympathetic nerve fibres. CRF2 receptors have recently been identified in dorsal root ganglia and the spinal cord (Million et al, 2006).

So far reports have identified the vagus to be the main pathway by which central CRF mediates reduced gastric emptying (Mönnikes et al, 1992; Lee and Sarna, 1997). This might also explain why the effect of CRF on small intestinal motility is minimal, since this gut region has significantly less vagal neural input than the stomach.

Colonic motor function changes induced by central CRF involve peripheral cholinergic nerves and CRF-1 receptors, which are known to be expressed in rodent colonic myenteric neurons (Mönnikes et al, 1993; Miampamba et al, 2002; Chatzaki et al, 2004a; Lui et al, 2005; Yuan et al, 2007). CRF projections from the Barrington nucleus (part of the locus coeruleus) innervate parasympathetic pre-ganglionic nerve fibres that synapse in the distal colon (Valentino et al, 2000), although some data indicates that peripheral sympathetic neural pathways may also be involved in CRF signalling (Nakade et al, 2005). CRF appears to affect colonic motor function via stimulation of neurons of the

colonic myenteric plexus. Data in man to support this is the report that rectal sensation and smooth muscle compliance are enhanced by exogenous peripheral CRF acting on rectal afferent nerve fibres (Lembo et al, 1996).

CRF seems to be involved in various levels of neural control, incorporating brain centres, the autonomic nervous system as well as the enteric nervous system itself. CRF is also secreted by nerves within the digestive tract (Porcher et al, 2006). It is therefore conceivable that it locally influences gut motor activity in this way, since colonic smooth muscle shows CRF receptors (Schäfer et al, 1997). Work by Lui et al (2005) has elegantly demonstrated in a guinea pig model, the expression of CRF1 receptor mRNA in the myenteric and submucosal plexuses in all regions of the small and large intestine, as well as the myenteric plexus of the stomach. Application of CRF evoked a depolarising response with excitability of myenteric and submucosal neurons, which could be suppressed by non-selective and CRF1-selective receptor antagonists. This is powerful evidence for CRF1-receptor mediated action of CRF in the enteric nervous system.

Further work by the same group (Lui et al, 2006) has shown that CRF immunoreactive nerve fibres are localised proportionately more in the colon than the upper gut, and that they occur in the circular muscle and submucosal arterioles as well. Additionally, cell bodies are more abundant in the myenteric plexus compared with the submucosal plexus. Ileal myenteric plexus CRF immunoreactive cell bodies expressed choline acetyltransferase, substance P and nitric oxide synthase. The submucosal ganglia expressed vasoactive intestinal peptide (VIP), and appeared to be secretomotor/vasodilatory neurons. CRF1 receptor immunoreactivity in the submucosal plexus additionally co-localised with expression of neuropeptide Y. Interestingly, CRF immunoreactive neurons did not express immunoreactivity for CRF1 receptor, while this receptor was expressed in neighbouring neurons, indicating that secretomotor neurons may synapse with their cholinergic neighbours.

This new evidence supports the putative role of CRF in gut motor function via neural mechanisms. However, the integrative role of CRF as a neuro-peptide appears to go beyond neural signalling and incorporates immune-inflammatory reactions as well. Because the gut has such a high concentration of immune-inflammatory cells, and because the gut lumen is an environment with such a high bacterial load, neuroimmunomodulation has particular significance in the gastrointestinal tract, as will be discussed in subsequent sections of this review.

1.3.4. CRF and immune-inflammatory modulation

The hypothesis that stress modulates gut immunoinflammatory mechanisms is worth pursuing. It is an idea that has only recently piqued the interest of researchers in the gastroenterological field and seems a logical line of investigation in the critically ill.

In contrast to adrenal glucocorticoids, hypothalamic hormones such as CRF are pro-inflammatory and potentiate immune function. Various studies have demonstrated that CRF is present in inflammatory sites, mainly within inflammatory cells (Karalis et al, 1991; Crofford et al, 1993; Mastorakos et al, 1995). CRF and/or CRF mRNA is also present in circulating white cells, and cells of the spleen or thymus (Stephanou et al, 1990; Aird et al, 1993; Ekman et al, 1993). It can increase the proliferation of T lymphocytes, and stimulate interleukin production from macrophages (Angioni et al, 1993). Therefore CRF has immune activity.

The exact mechanism by which CRF induces inflammation is not fully known. Certainly immune cells in general are responsive to neuropeptides (Van Hagen et al, 1999). It is apparent that CRF released during stress does affect gastrointestinal sensory and inflammatory processes via direct activation of gut neurons and immune cells (Castagliuolo et al, 1996b; Gué et al, 1997; Taché et al, 1999). The various kinds of immune/inflammatory cells in the bowel wall (lymphocytes, macrophages, polymorphonuclear leucocytes and mast cells) are involved in paracrine signalling with the enteric nervous system (Wood et al, 1999). The signalling between neurons and mast cells is the most widely described and perhaps the best understood.

1.3.4.1. CRF and mast cells

Mast cells have pro-inflammatory effects and can alter barrier and transport properties, and play a role in regulation of mucosal physiology. Mast cells respond to antigen, bacterial toxin, neurotransmitters and other IgE-independent stimuli. The increased intestinal permeability associated with the release of specific mast cell mediators such as histamine, cytokines and tryptase, is a result of increased gut inter-cellular tight junction permeability (Hirase et al, 2001). Both small and large molecules are affected via the paracellular route (Yu and Perdue, 2001).

Mast cells have been implicated in ischaemia-associated damage to the intestinal mucosa. Ischaemia-reperfusion is reportedly associated with significant morphological damage to the mucosa, along with changes in permeability (Szabó et al, 1997). In this experiment on dogs, mast cell stabilisers were able to significantly blunt the hyper-permeability following intestinal ischaemia, but did not really influence the associated structural injury to the mucosa. Later, at the same institution, Boros et al (1999) showed that pre-treatment with mast cell stabilising agents caused exhaustion of mast cells prior to the ischaemic insult. As in the previous study, this pre-injury depletion of mast cells was associated with a decrease in the extent and severity of histological damage to the mucosa. In humans, Santos et al (1998) showed that acute stress caused enhanced ion and water secretion in the jejunum, and that the jejunal secretory fluid contained highly specific mediators released as a result of mast cell degranulation, such as human mast cell tryptase. Mast cell tryptase has also been detected in intestinal juices collected from Irritable Bowel Syndrome patients with the combination of enhanced background life stress and an acute cold pain stressor. This occurs together with epithelial

functional changes in the jejunum indicative of barrier defects (Alonso et al, 2008). Recent work has shown that in a model of low-grade inflammation, mast cell infiltration accompanying intestinal nematode infestation resulted in suppression of tight junction protein mRNA expression, and portal vein appearance of an endotoxin load delivered into the gut (Farid et al, 2008). Therefore, gut permeability induced by different stressors appears to share a common mast cell related pathogenic mechanism.

Chronic stress causes defects in the epithelial barrier function of the gut associated with massive mucosal mast cell hyperplasia and enhanced degree of activation (Santos et al, 2001). In this research, administration of CRF was able to mimic the intestinal permeability associated with mast cell infiltration. A fascinating discovery is that mast cells themselves synthesise and produce CRF (Kempuraj et al, 2004). The same research group went on to demonstrate the expression of functional CRF receptors on mast cells (Cao et al, 2005). Indeed mast cells can be induced to degranulate by CRF via a CRF-receptor mediated mechanism, and are therefore CRF targets (Theoharides et al, 2004). The receptor-mediated effect of CRF to induce secretions from the mast cells can be blocked using CRF1-receptor but not CRF2-receptor blockade. This suggests that CRF may act in a paracrine and autocrine manner via CRF1 receptors on mast cells. The resulting secretory products of mast cells are inflammatory and - in the gut - result in disturbances in mucosal barrier and transport physiology, which can be blocked by both CRF antagonists and mast cell stabilisers (Cooke et al, 1993; Santos et al, 1999; Santos et al, 2008; Wallon et al, 2008). Large molecule gut permeability in piglets stressed by premature weaning is preventable by mast cell stabilisation, and is mediated via peripheral signalling pathways involving CRF (Moeser et al, 2007a; Moeser et al, 2007b). Chronic (12 days) subcutaneous CRF delivery induces mast cell hyperplasia together with colonic barrier defects in wild-type animals which are not reproducible in mast cell deficient animals (Santos et al, 1999; Teitelbaum et al, 2008). Thus, both CRF and mast cells are involved in the mechanics of intestinal permeability.

CRF, however, is a most critical mediator of this response. Using RNA interference to silence CRF expression in the wall of the rat ileum, La Fleur et al (2005) showed that the local synthesis of CRF was essential for the modulation of gut inflammation and motility. Recently, in animals, CRF was shown to promote selective release of certain mast cell products in a receptor-mediated fashion, which resulted in enhanced gut permeability via a paracellular route (Barreau et al, 2007; Santos et al, 2008). Other current work has further demonstrated that CRF acts in the gut in a receptor-mediated fashion in concert with mast cells. Using selective CRF receptor agonists/antagonists in wild type and mast cell knock-out mice, Teitelbaum et al (2008) showed that both CRF-1 and CRF-2 are implicated in barrier dysfunction. In their experiments, however, CRF receptor agonists did not produce barrier dysfunctions in mast cell deficient mice, once again reinforcing the integral relationship between CRF and mast cells in gut barrier effectiveness. Data from in vitro studies on human colonic tissue support

this finding. Using colonic biopsy specimens from healthy volunteers, Wallon et al (2008) demonstrated in a controlled experiment that exposure of biopsies to CRF resulted in mast cell activation and that mast cells expressed both CRF-1 and CRF-2 receptors.

1.3.4.2. Mast cells and enteric nerves

Local mucosal inflammation of any kind, even if mild, superficial ("sub-clinical") or transient in nature, can alter neuromotor function in the gut and this effect is complex and seems to involve bi-directional interactions between immune cells, smooth muscle and enteric neural apparatus. Furthermore, changes in neuromuscular function occur at remote, non-inflamed sites in response to localised inflammation elsewhere in the gut stressing the probability that intrinsic and extrinsic neural circuitry is influenced (de Jonge et al, 2003; Schwarz et al, 2004).

Nerves and mast cells both seem to participate in the epithelial dysfunction associated with stress, and it is now known that gut mast cells are innervated directly by projections from the central nervous system (Ottaway, 1991; Stead, 1992; McKay and Bienenstock, 1994; Wood et al, 1999). It has been shown in resected human small and large intestine that nerve-mast cell interactions regulate ion transport, possibly because released neurotransmitters amplify mast cell-regulated effects (Crowe and Perdue, 1993). More than one study has shown that neuropeptides from nerves can elicit release of inflammatory mediators from mast cells (Shanahan et al, 1985; Shanahan and Anton, 1988). Some work (Suzuki et al, 1999) has shown that degranulation of mast cells is as a result of direct contact of mast cells with activated nerve fibres (rather than via an intermediary cell). In resected human tissue, over 75% of mast cells are shown to be in close apposition to nerve fibres (Stead et al, 1989).

Altered output of central stress circuits seem to profoundly influence gut systems in this regard - gut immunity is shifted from Th1 to Th2 response, showing a greatly increased mast cellularity (Elenkov and Chrousos, 1999). While mast cells are required for the response, stress-induced ion and macromolecule hyper-permeability and ionic hyper-secretion seem to be mediated via a parasympathetic cholinergic neuroactive substance. As atropine can prevent the hyper-permeability (Saunders et al, 1997; Kiliaan et al, 1998), this neuroactive substance is probably acetylcholine, but enteric nerves also release non-classical neurotransmitters such as VIP, substance P, nitric oxide and 5-HT and many other neuropeptides including CRF (Cooke, 1986; Castagliuolo et al, 1998; Lui et al, 2006). These neurotransmitters and neuropeptides activate mast cells and have pro-secretory end-effects.

Since CRF receptors have been identified in the spinal cord and mast cells are found throughout all gut layers, it is difficult to comment on the amplitude of a CRF effect on immune-inflammatory changes in the gut. In fact the cellular origins of CRF and the related ligands that bind to CRF receptors in the periphery are not at all clear as yet.

Having reviewed the published evidence on the general role of CRF in stress-related gastrointestinal dysfunction, questions emerge as to whether CRF can be implicated in the characteristic functional impairments of the gut observed in the ICU. Patients with traumatic injury and shock exhibit a profound CRF-driven stress response, yet it is unknown how high levels of CRF release may impact upon gastrointestinal compromise in this patient group, or whether CRF released in intestinal tissue is involved. The broad aim of this study was, therefore, to investigate whether CRF plays any role in alterations in acute post-operative gastrointestinal function in critically ill adults following traumatic abdominal injury. The specific questions addressed in this thesis are:

1. What is the nature and degree of alterations in acute post-operative gastrointestinal dysfunction in this patient group?
2. Is there an association between circulating plasma CRF and gastrointestinal dysfunction?
3. Is CRF detectable in small intestinal tissue following traumatic abdominal injury, and is it locally produced in the intestine?
4. Is there an association between CRF in small intestinal tissue and gastrointestinal dysfunction?
5. Is there an association between CRF in small intestinal tissue and mast cells; and between mast cells and gastrointestinal dysfunction?

1.4. Aim

To investigate the role of CRF in alterations in acute post-operative gastrointestinal function in critically ill adults with traumatic abdominal injury compared with elective surgical patients.

1.5. Objectives

a) To measure and compare plasma CRF levels in critically ill adults with traumatic abdominal injury and a reference group of patients undergoing elective abdominal surgery with bowel resection.
b) To measure and compare tissue CRF peptide levels in resected bowel tissue in the two patient groups.
c) To measure and compare post-operative small intestinal permeability in the two patient groups.
d) To measure and compare the rate of post-operative gastric emptying in the two patient groups.
e) To determine whether bowel tissues synthesise CRF by measuring mRNA expression in resected bowel tissue obtained from the two groups.
f) To quantify mast cell presence in resected small bowel tissue and establish whether there is an association between mast cell numbers and tissue CRF levels.
g) To determine whether any of these above measures can be related to clinical course and outcome.

Chapter 2: Procedures and Methods

2.1 Study design

This was a prospective, investigational clinical study comparing CRF levels and gut function in patients with traumatic shock with a stable surgical control group.

2.2. Study populations

Subjects were recruited from patients admitted to the Department of Surgery, Groote Schuur Hospital and University of Cape Town, South Africa for abdominal surgery.

2.2.1. Trauma patients

The trauma group comprised shocked, traumatically injured adult patients admitted to the trauma unit. All patients that were older than 18 years and who met the predefined inclusion criteria were considered eligible to participate.

Inclusion criteria were:

a) penetrating or blunt abdominal trauma accompanied by shock and necessitating resuscitation. Shock requiring resuscitation was defined as follows:

- a systolic blood pressure of < 90 mmHg and/or
- acidosis and/or
- a urine output of <1 ml/kg/hr and/or
- the need for >2 litres of intravenous fluids during resuscitation.

b) requirement for emergency laparotomy where surgical resection and anastomosis involving the small bowel could be reasonably expected, based on the injury

c) entry to the trauma unit within 12 hours of injury

Immediate exclusion criteria were:

a) no immediate foreseeable prognosis (i.e. the person was judged unlikely to survive 24 hours or had severe brain injury)

b) pregnancy or lactation

c) known inflammatory bowel disease

Secondary exclusion criteria were:

a) lack of informed consent

b) a finding at laparotomy of significant previous intestinal surgical resection

c) partial or total gastrectomy at the current or at a previous surgery.

2.2.2. Elective patients

The elective surgery group comprised stable adult patients undergoing elective hepato-biliary or pancreatic surgery at the same university hospital. All eligible patients over 18 years of age were screened for participation.

Inclusion criteria were:

a) Obstructive lesion in head of pancreas or pancreatic duct OR
b) Common bile duct injury, obstruction, stricture or other lesion.

In either case a Whipple's procedure or Frey procedure or hepatico-jejunostomy or other similar hepato-biliary surgery involving small intestinal bypass loop or some form of entero-enterostomy was required.

Exclusion criteria were:

a) lack of informed consent
b) pregnancy or lactation
c) inflammatory bowel disease
d) Previous significant intestinal resection
e) Partial or total gastrectomy at the current or at a previous surgery

2.3. Ethical considerations

2.3.1 Ethical approval to conduct research

The study protocol was approved by the Human Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (ref no: 358/2003). No patients were enrolled into the study until the protocol, Patient Information and Consent Sheet and data collection/documentation material were approved in writing by the Ethics Committee. The study was performed in accordance with the principles of the Declaration of Helsinki (World Medical Association, 2000), ICH (EMEA, 1997) and South African (SA DoH, 2000) Good Clinical Practice (GCP) and the laws of South Africa. The study was covered by the insurance policy held by the University of Cape Town.

2.3.2. Informed consent

Written informed consent was obtained from all patients prior to inclusion or, in the case of trauma patients, their next of kin prior to commencement of trial procedures. The protocol required the carefully timed taking of blood and tissue samples in the immediate period following acute injury and/or surgery. In the case of those trauma patients, where consent could not be obtained prior to these procedures being performed, informed consent was requested from the subject or his next of kin before biological specimens were used. If the patient did not consent, the samples were destroyed

(see 2.3.4.). Prior approval for this mechanism of obtaining consent was granted by the Ethics Committee before research began. Such procedures are acceptable if approved by the Ethics Committee according to paragraph 4.8.15 of the ICH GCP guideline (EMEA, 1997), which states,

> "*In emergency situations, when prior consent of the subject is not possible, the consent of the subject's legally acceptable representative, if present should be requested. When prior consent of the subject is not possible, and the subject's legally acceptable representative is not available, enrolment of the subject should require measures described in the protocol and/or elsewhere, with documented approval/favourable opinion of the IRB/EC, to protect the rights, safety and well-being of the subject and to ensure compliance with applicable regulatory requirements. The subject or the subject's legally acceptable representative should be informed about the trial as soon as possible and consent to continue and other consent as appropriate should be requested".*

No elective surgery patient entered the study without signing an informed consent form after a full and adequate oral and written explanation of the study was provided by the investigator prior to all study procedures. Patients maintained the right to withdraw from the trial at any stage without stating a reason.

2.3.3. Confidentiality and anonymity

The data generated from the trial was codified and stored in a computer database in a secure facility, in a manner that maintained patients' confidentiality and anonymity. For data verification and quality control purposes, regulatory authorities and/or members of the Ethics Committee could be allowed access to patient data under conditions of strict confidentiality. This was stated in the Patient Information Sheet. The anonymity of participants will continue to be guaranteed in any publication of the data.

2.3.4. Removal of biological specimens (human tissue and blood)

Tissue samples were only taken from resected tissue that was specifically intended for incineration/destruction. The researcher had no role in the surgical decision-making process. The various surgeons acted independently based on their clinical judgement, experience and expertise. No surgeons were co-investigators on the study. Tissue samples were coded and could not be independently identified. Biological specimens (tissue and/or blood) obtained from trauma patients who later did not give consent for the study were immediately destroyed and any derived data eliminated. Biological specimens obtained for the purpose of the trial were used only for the stated scientific investigations indicated in the study aims.

2.4. Demographic and clinical data

Demographic data was recorded for all patients at study entry. Relevant clinical data was recorded as described below.

For the **trauma group** this included:

At baseline:

- Nature and detailed description of the injury/injuries
- Injury Severity Score (Baker et al, 1974) calculation
- Baseline vital signs and indicators of shock
- Time delay between injury and presentation to hospital
- Time delay between injury and first emergency surgical procedure
- Time of induction of anaesthesia
- Volume and type of all intravenous fluids, including blood products
- Identity, route and dosage of all pharmacological substances administered
- Detail of the emergency operative procedure(s) performed
- Duration of surgery
- APACHE II score (Knaus et al, 1985) based on clinical data from the first 24 hours in the ICU

Daily for duration of study period:

- Vital signs
- Volume and type of all intravenous fluids, including blood products
- Identity, route and dosage of all pharmacological substances administered
- Details of further surgical procedures
- Indicators of gastrointestinal function including number of vomits, number and consistency of stools, gastric residual volume, and tolerance and progress of oral and/or enteral feeding. Diarrhoea was defined as 3 or more loose stools in a single 24 hour period. Constipation was defined as the passage of hard stool less than 3 times per week.
- Mode of ventilatory support (if any)
- Development of infectious complications
- Development of organ failure
- Medical interventions for the treatment of clinical complications

Outcome:

- Days in ICU
- Days on mechanical ventilation
- Days in hospital
- ICU mortality
- Hospital mortality

Similarly, for the **elective surgery group**:

At baseline:

- Diagnosis
- Time of induction of anaesthesia
- Time of commencement of surgery
- Volume and type of all intravenous fluids, including blood products
- Identity, route and dosage of all pharmacological substances administered
- Detail of elective operative procedure(s) performed
- Duration of surgery
- APACHE II score (Knaus et al, 1985) based on clinical data from the first 24 hours in the ICU

Daily for duration of study period:

- Vital signs
- Volume and type of all intravenous fluids, including blood products
- Identity, route and dosage of all pharmacological substances administered
- Details of further surgical procedures
- Indicators of gastrointestinal function including number of vomits, number and consistency of stools, gastric residual volume, and tolerance and progress of oral and/or enteral feeding.
- Mode of ventilatory support (if any)
- Development of infectious complications
- Development of organ failure
- Medical interventions for the treatment of clinical complications

Outcome:

- Days in ICU
- Days on mechanical ventilation
- Days in hospital
- ICU mortality
- Hospital mortality

2.5 Physiological methodology

2.5.1. Gastric emptying

Gastric emptying is measured by the rate of absorption of a loading dose of paracetamol into the blood, since paracetamol is only absorbed from the duodenum. The rate of appearance of paracetamol in peripheral blood, as well the maximum overall concentration of paracetamol measured during the test period and the time point at which that occurred can be used as indicators of the rate of gastric emptying.

Method

The patient was in a fasted state post-operatively, and had not received paracetamol for at least 24 hours prior to the test. An indwelling venous cannula was placed by aseptic technique in the cephalic/antecubital vein of the forearm to allow for multiple blood sampling, if one was not already in place. A baseline venous blood sample was drawn into a plain tube. Thereafter 1500 mg paracetamol (Panado®, AI Self Med, South Africa) was taken by mouth by the patient or was crushed and injected into the stomach via the existing nasogastric tube, and chased with 30-50 ml of tap water. Thereafter a 1 ml venous blood sample was drawn into a plain tube every 30 minutes for 3 hours. The specimens were processed within 1 hour of being drawn by centrifuging at 3000 rpm at room temperature for 10 minutes. The serum was drawn off and stored at -20ºC until analysis. Paracetamol concentrations were determined by the accredited laboratory of the Division of Pharmacology, Department of Medicine, University of Cape Town. The assay used (AxSYM™ Acetominophen Assay Reagent Pack, Abbott Laboratories, IL, USA) is based on Fluorescence Polarization Immunoassay (FPIA) technology. Since paracetamol is absorbed only from the duodenum, the area under the paracetamol versus time curve is an indication of gastric emptying, which is expressed as µg/ml x 3 hours. Area under the curve (AUC) was calculated using the linear trapezoid rule. The maximum concentration (C_{max}) of paracetamol as well as the time at which the C_{max} was measured (T_{max}) were also recorded as indicators of gastric emptying.

2.5.2. Intestinal permeability

The relative permeability of the small intestine can be measured by a combined sugar test. An oral loading dose of a non-hydrolysable disaccharide (lactulose) is given together with a monosaccharide (mannitol). Lactulose is not hydrolysed in the intestinal tract and cannot be taken up by physiological transcellular absorption pathways. However, the intact molecule will pass the mucosal barrier via paracellular routes if permeability of the small intestine is increased. Lactulose absorbed in this way can be detected unchanged in the urine. Mannitol, however, is absorbed via transcellular routes and is thus used as a reference for normal absorption. There are both pre-mucosal and post-mucosal factors which affect the urinary excretion of these two sugars (e.g. gastric and intestinal dilution and blood flow, and renal function among many others). However, these factors affect the two probe sugars in an identical manner. The differential ratio is therefore a valid marker. Once the sugars have permeated the gut mucosa, metabolism of these molecules does not take place. They are therefore excreted intact in urine. The index of intestinal permeability is the ratio of the concentration of the two sugars measured in a known volume of urine collected during a specified period following the combined loading dose of the two sugars.

Method

Mannitol and lactulose were both withheld from the patient during the 24 hours prior to the test. A baseline urine specimen was taken as a reference sample just before the sugars were given. The urinary catheter bag was then emptied and the urine contained therein discarded. With the patient in the fasting state, 10 g lactulose (Lacson®, Aspen Pharmacare) and 5 g mannitol (Intramed Mannitol®, Bodene) were taken together by mouth in a small amount of tap water, or were injected into the stomach via the existing nasogastric tube. Thereafter urine was collected for 6 hours. The 6-hour urine collection was accurately measured for volume and a sample taken for analysis. All specimens were preserved by adding 1 ml 0.1% sodium azide to the specimen. Samples were stored at -20°C until analysis. The concentrations of urine lactulose and mannitol (mg/l) were measured simultaneously by HPLC at the Esoteric Sciences Laboratory of the commercial Ampath Pathology Laboratories (Drs Du Buisson, Bruinette, Kramer Inc, SA). The test result is expressed as the ratio of urinary lactulose to mannitol concentrations, as well as the percentage of the orally administered load of each sugar that was recovered in the urine.

2.6. Biological specimen sampling procedures

2.6.1. Blood sampling

Method

In both groups, prior to induction of anaesthetic, 5 ml of venous blood was drawn by aseptic technique into a cool EDTA tube to which cold aprotinin (Trasylol®, Bayer AG, Germany) was added (equivalent to 0.6T IU/ml of blood). Filled tubes were gently rocked by hand to inhibit proteinase activity. Specimens were then centrifuged at 1600 x g for 10 minutes at room temperature and the plasma was taken off. Plasma samples were stored at -70ºC without additives until analysis. In both groups, blood sampling was repeated as described above at the moment of surgical resection of the bowel, and at the time of the physiological tests described above, on the first post-operative day.

2.6.2. Tissue sampling

Method

In the trauma patients, a 1 cm^3 piece of resected, macroscopically normal, full thickness small bowel adjacent to the gunshot wound (but not so close as to be blast-injured) was collected in the operating theatre immediately upon resection. In elective patients, a similar small bowel specimen was obtained from the enterostomy site or the end of the jejunal loop during Whipple's, Frey or hepatico-jejunostomy procedures. In both groups the tissue was divided with a clean sterile scalpel blade and one half immediately placed into a sterile RNase free polypropylene disposable tube (Axygen Scientific, USA) and flash-frozen in liquid nitrogen. Thereafter it was stored in liquid nitrogen for 24 hours and then transferred to an ultra-low temperature freezer until CRF extraction and analysis.

2.7. Peptide extraction methods

2.7.1. Peptide extraction from plasma

Method

For analysis, samples were thawed on ice and immediately acidified with 1% trifluoroacetic acid (TFA) in water, in a ratio of 1:1. Samples were gently mixed by manual inversion and centrifuged at 17000 x g for 20 minutes at 4ºC. The supernatant was removed for analysis. The acidified plasma was then loaded onto a C18 cartridge, which had been pre-equilibrated by washing once with 1 ml 60% acetonitrile in 1% TFA followed by 3 washes each with 3 ml 1% TFA. The C18 cartridge loaded with the acidified plasma solution was then washed twice with 3ml 1% TFA without applying pressure to the column. Both times the wash was discarded. The peptide was then eluted slowly with 3 ml 60% acetonitrile in 1%TFA. The eluate was collected in a polypropylene tube and frozen at -80°C. Once frozen, samples were lyophilised by placing in a freeze dryer overnight. The lyophilised powder was stored at -20ºC until CRF quantification.

2.7.2. Peptide extraction from tissue

Method

Tissue of approximately the same size was used to perform the peptide extraction from each specimen. The specimen was thawed on ice. Immediately 1 ml of 0.1 M acetic acid was added to each tissue sample, which was then transferred to a boiling water bath for 10 minutes. Samples were transferred to ice and cooled after which each specimen was manually homogenised in a polypropylene tube using 15 strokes with a glass plunger. A separate, clean plunger was used for each specimen. Each homogenate was then centrifuged for 15 minutes at 13000 x g, the supernatant taken off and 150 µl of each specimen was set aside for total protein analysis. The remainder was frozen at -80ºC and then lyophilised by placing in a freeze dryer overnight. The lyophilised powder was stored at -20ºC until CRF quantification.

2.8. Laboratory methodology

2.8.1. Radio-immunoassay for CRF peptide

The assay works on the principle of competitive binding of a radioisotope labelled ligand and an unknown (or standard) ligand to a limited amount of antibodies specific to that ligand. As the amount of unknown peptide in the sample increases, less labelled ligand is able to bind to the specific antibodies. The antibody-bound ligand is then separated from free peptide. In this method, this is achieved by adding a second antibody directed against the first, which allows precipitation of the ligand-antibody complexes. These complexes are separated from free ligand in the supernatant fluid. The radioactivity of each is then measured. The amount of radiolabelled ligand is inversely proportional to the amount of unknown ligand in the sample. After determining the ratio of bound to unbound ligand, the concentration of unknown ligand in the sample can be read from a standard curve created using known standard peptide concentrations.

Method

Lyophilised extracts from plasma and tissue specimens were analysed in duplicate for the level CRF peptide using a RIA kit (Corticotropin-releasing factor (CRF), human, RIA kit, Phoenix Pharmaceuticals Inc, USA). Clean disposable gloves were worn for the procedure. As described by the manufacturer, the kit shows no cross-reactivity with human ACTH or with rat or human urocortin, and has a sensitivity of 10 pg/ml. No positive controls were used because the available reagents supplied were only sufficient to create the standard curve and assay the unknown specimens, all in duplicate.

The RIA buffer stock solution was diluted with 150 ml sterile distilled water. Using polystyrene tubes, the standards were made up as follows. The standard peptide was reconstituted with 1 ml of RIA buffer, mixed well and stored on ice. This was the stock solution. Using a 1% dilution of the stock solution (designated standard 0), further dilutions for the standards (A-H) were made according to the protocol provided with the kit (see Table 2.1 reproduced with minor adaptations from the kit protocol provided).

Table 2.1. Preparation of standard dilutions

Standard	Sample	RIA Buffer	Standard Concentration
0	10 µl of stock	990 µl	128000 pg/ml
A	10 µl of 0	990 µl	1280 pg/ml
B	500 µl of A	500 µl	640 pg/ml
C	500 µl of B	500 µl	320 pg/ml
D	500 µl of C	500 µl	160 pg/ml
E	500 µl of D	500 µl	80 pg/ml
F	500 µl of E	500 µl	40 pg/ml
G	500 µl of F	500 µl	20 pg/ml
H	500 µl of G	500 µl	10 pg/ml

Before continuing with the assay procedure, the primary antibody (rabbit anti-CRF) was reconstituted in 13 ml RIA buffer, mixed and stored on ice. Unknown samples were reconstituted in 500 µl RIA buffer and mixed well to fully dissolve the lyophilised powder.

The assays for all standards and unknowns were carried out in duplicate. For both standard peptides and reconstituted unknown samples, 100 µl was pipetted into pre-labelled tubes. Then 100 µl of the primary antibody was added to each tube, which was vortexed. The tubes were then covered with foil and incubated at 4°C for 20-24 hours. After 24 hours, the ^{125}I-peptide tracer was reconstituted with 13 ml RIA buffer and mixed well by hand. The ^{125}I concentration was checked by counting for 1 minute in

the gamma counter, and adjusted with RIA buffer to 8000 – 10 000 cpm/100 µl total activity. Then 100 µl of ^{125}I-peptide tracer solution was added to each tube, which was vortexed, covered and incubated at 4°C for another 20-24 hours. After the second 24 hour incubation period, the secondary antibody (goat anti-rabbit IgG serum) and the normal rabbit serum (unlabelled ligand) were each reconstituted with 13 ml RIA buffer. Of each, 100 µl was added to all tubes. Samples were then vortexed and incubated at room temperature for 90 minutes. Thereafter 500 µl of RIA buffer was added to each tube. Tubes were first vortexed then centrifuged at 1700 x g for 20 minutes at 4°C. The supernatant was decanted by hand and discarded and remaining liquid in each tube was removed by gentle inversion of the tube onto a paper towel. The counts per minute (cpm) of the pellet in each tube was counted immediately on the gamma counter.

As part of the assay duplicate control tubes were set up for non-specific binding and total binding. For the non-specific binding, 200 µl of RIA buffer was pipetted into a labelled tube. At the same time, for total binding 100 µl of RIA buffer and 100 µl primary antibody were added to duplicate tubes. Both sets of tubes were vortexed, covered and incubated for 20-24 hours at 4°C along with the standards and unknown samples. Thereafter, both total binding and non-specific binding tubes were treated in the same way as the standards and unknown samples. After the first incubation 100 µl of the ^{125}I-peptide tracer was added to all tubes, which were vortexed and incubated for a further 20-24 hours at 4°C. Then 100 µl each of the secondary antibody and the normal rabbit serum (unlabelled ligand) were added. Samples were then vortexed and incubated at room temperature for 90 minutes. Thereafter 500 µl of RIA buffer was added to each tube. Tubes were first vortexed then centrifuged at 1700 x g for 20 minutes at 4°C. As with standard and unknowns, the supernatant was decanted by hand and discarded. Remaining liquid in each tube was removed by gentle inversion of the tube onto a paper towel. The counts per minute (cpm) of the pellet in each tube was counted immediately on the gamma counter.

Calculations:

1. The mean cpm for the duplicate non-specific binding tubes was calculated = NSB
2. The mean cpm for the duplicate total binding tubes was calculated = TB
3. B_0 (zero binding) is determined using the equation B_0 = TB-NSB
4. For each duplicate standard sample determine the percentage binding (B/B_0 (%))using the equation:

$$B/B_0\ (\%) = \frac{[\text{mean cpm standard} - \text{NSB}] \times 100\%}{B_0}$$

5. On graph paper the standard curve was plotted by plotting the B/B_0 (%) on the y-axis and the log of the concentrations of the standards on the x-axis in reverse order i.e. H to A. The B/B_0 (%) for

each standard was then plotted relative to its known peptide concentration. A best fit curve was constructed.

6. For each duplicate unknown sample the mean cpm was calculated and the percentage binding (B/B_0 (%))was determined using the equation:

$$B/B_0\ (\%) = \frac{[\text{mean cpm standard} - \text{NSB}] \times 100\%}{B_0}$$

7. Using the B/B_0 (%) calculated for each unknown sample the concentration of peptide in the assayed sample was interpolated from the standard curve.

CRF in plasma specimens was expressed pg/ml after accounting for dilution factors. CRF in tissue specimens was expressed as a percentage of the total protein for each specimen, which was quantified as described below.

Batch normalisation

The blood samples obtained from the study patients were analysed in 2 batches using 2 separate RIA kits. On analysing the data a batch difference became apparent. The reason for this batch difference was that the radioactive tracer (I^{125}) included in the second kit had decayed, and was reaching expiry when the assay was run. The result was that CRF concentrations measured using this kit were half of those obtained from the first kit for the same percentage binding, because of a left shift in the standard curve. This is illustrated on the standard curves, which were drawn as the best fit by hand, and are shown in Figure 2.1. below.

The number of unknown study samples to be analysed required all the reagent provided in the kits. Therefore it was not possible to run internal positive controls and obtain a coefficient of variation between the two assays. However, the data did require normalisation in order to statistically analyse the results from the two batches as one data set. This was particularly important since most of the trauma samples were assayed in the first batch and many of the elective samples in the second batch. Since the objective was to perform an inter-group comparison the results had to be combined.

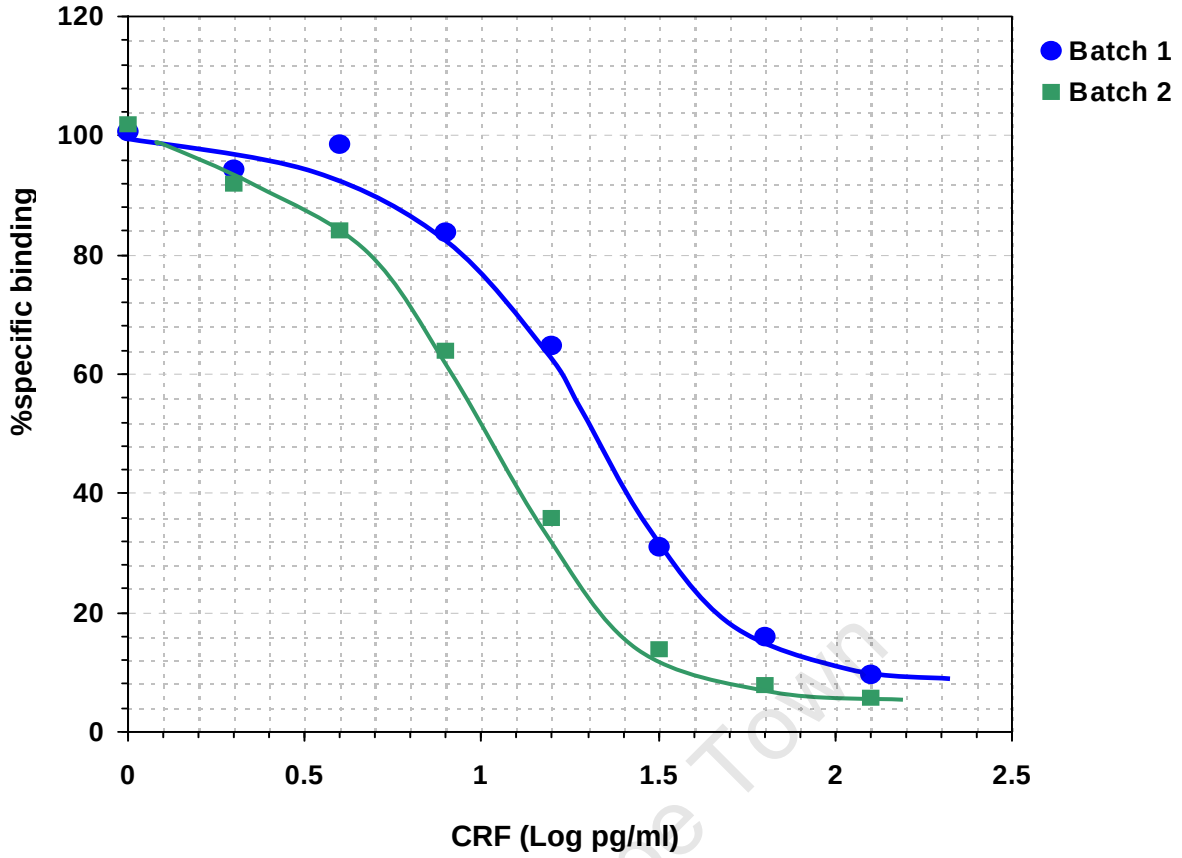


Figure 2.1. Best fit standard curves for RIA batches

Radioactive I^{125} decays with a physical half-life of 60 days. It is assumed that energy released during radioactive decay causes damage to the ligand so that binding is impaired, while specific radioactivity remains the same. The concentration of the iodinated ligand in the stock solution can be calculated according to the equation:

Concentration of radioligand (pM) = counts per minute x specific activity / volume in ml

Therefore, using the specific activity of I^{125} of 2190 Ci/mmol and the counts per minute in 100µl of stock solution obtained for each kit, the concentration of radioligand in the decayed batch could be exactly determined. The concentration of labeled ligand in the second batch was found to be 65% of the first. When studying the standard curves the following can be observed. Firstly, the curves are the same shape with the linear portion having parallel gradients. Secondly, for any percentage specific binding, reading off the second batch curve (in green) gives a log [CRF] of 66% that of the first batch (standard curve in blue). The antibody binding between radio-labeled and standard peptide is competitive, and the amount of binding of radio-labeled ligand is inversely proportional to the amount of unlabelled standard peptide in the solution. The standard curve is thus derived from the percentage competitive binding of radioisotope tracer versus a known amount of standard unlabelled peptide. The unknown amount of peptide in a patient specimen being assayed can then be derived from the standard curve using the percentage specific binding obtained from the gamma counts of unknown

samples. In the case of a reduction in the concentration of radioactive ligand, as occurred with the second batch, one would expect a left-shifted standard curve since proportionately more standard peptide is available to bind competitively. Therefore, the left shift in the standard curve in batch 2 can be accounted for by the 65% reduction in concentration of radioisotope labeled ligand. In order to combine the data from the two batches, the results from the second batch were uniformly adjusted by this factor.

2.8.2. Total protein quantitation

Method

Total protein content of the extract of tissue specimens was assayed using the colorimetric protein assay kit (BCA™ Protein Assay Kit, Pierce, USA). Using 96 well ELISA plates, 25µl of each unknown sample was pipetted out in duplicate along with 25µl of bovine serum albumin (BSA) standards for the standard curve. See Table 2.2 below.

Table 2.2 Standard concentrations

Standard	Concentration of BSA (ug/ml)
A	2000
B	1500
C	1000
D	750
E	500
F	250
G	125
H	25
I	0

Then 200µl of 1:50 dilution working reagent was added to each well. The plates were covered and incubated at 37°C for 30 minutes in an oven. After cooling to room temperature the plates were read on an ELISA reader at an absorbance of 562 nm. The average absorbance of the blank standard sample was subtracted from the other standards and the unknown samples. The standard curve was constructed by plotting the blank-corrected absorbance for each standard sample on the y-axis against the known concentration of the each standard on the x-axis. Total protein values for unknowns were interpolated from the standard curve and expressed in µg/ml after accounting for dilution factors.

2.8.3. RNA extraction from tissue

RNA is unstable in comparison to DNA. This is due to the presence of highly stable and active ribonuclease enzymes (RNAses), which are very effective at breaking down RNA. For optimal results, the RNA sample should be of high quality and purity, free of contaminating DNA, and protected from RNAse activity. The sampling of biological specimens must therefore incorporate steps to prevent contamination of samples with RNAses from the environment, and to immediately stabilise the RNA during the storage process so that the expression pattern is preserved. In order to extract RNA from tissues it is essential to properly disrupt all cell and organelle membranes to release all RNA and achieve a good recovery. This is especially true where the abundance of contractile proteins, collagens and connective tissue (such as in gut) makes RNA extraction challenging. Beyond disrupting the cellular material, the sample requires homogenisation to shear high molecular weight components of the tissue and genomic DNA. Good homogenisation produces a less viscous lysate and allows for superior RNA yields. The isolation of the RNA (after the method of Chomczynski and Sacchi, 1987) then utilises a mixture of guanidine thiocyanate and phenol-chloroform to simultaneously denature endogenous endonucleases and phase separate the RNA from protein and DNA in the specimen. The RNA can then be precipitated in isopropanol, washed and resuspended in preparation for analysis. The quality and integrity of the RNA can be determined by the A260/A280 ratio and good separation of the 28S and 18S ribosomal RNA bands on gel electrophoresis.

Method

During the RNA extraction procedure, clean disposable gloves were worn, and sterile pipette tips were used. Tissue specimens of approximately 100 mg were taken from those originally flash-frozen upon surgical resection and stored at -80ºC. For RNA extraction, tissue was taken directly from the freezer into a sterile, RNAse-free tube (Axygen Scientific, USA). Three methods of breaking up the tissue were used experimentally to determine which yielded the best results. Firstly, using a Polytron® (Kinematica, Switzerland), tissue samples were homogenised in 1 ml of TriPure™Isolation Reagent using 3 cycles of 10 seconds each interrupted by a brief break period of 2 seconds. Secondly, frozen tissue samples were immersed in TriPure™Isolation Reagent under liquid nitrogen and crushed in a DEPC-treated autoclaved pestle and mortar before being homogenised using a Polytron®. Thirdly, frozen tissue specimens were placed into a sterile, RNAse-free cryovial containing 1 ml of TriPure™Isolation Reagent together with a stainless steel DEPC-treated and autoclaved 10 mm ball. Each specimen was homogenised by fitting the cryovial into a mechanical power homogeniser and oscillated for 30 seconds. Each 30s cycle comprised 5 cycles of 4 seconds each of oscillatory motion interrupted by a 2 second motionless rest phase to prevent overheating of the specimen. All methods broke up the tissue to the extent that no visible pieces of tissue remained. The homogenate was transferred into a sterile, RNAse-free, disposable polypropylene tube, pulsed and left to stand at room temperature to ensure complete dissociation of the nucleoprotein complexes.

Then 200 µl of cold pure chloroform was added to each specimen, which was vortexed vigorously for 20 seconds. Specimens were then left to incubate for 15 minutes at room temperature whereafter they were centrifuged at 12000 x g for 15 minutes at 4°C to phase separate the solution. From the upper aqueous phase, 400 µl was removed and transferred into a new sterile polypropylene centrifuge tube. RNA was precipitated from the aqueous phase by adding 500 µl of cold isopropanol and shaking the tube by hand inversion to mix. The sample was then incubated for 1 hour at room temperature or overnight at -20°C to allow the RNA to fully precipitate. The specimen was centrifuged again at 12000 x g for 10 minutes at 4°C and the supernatant discarded. The pellet was washed by adding 1 ml of cold 75% ethanol to the tube and vortexing. The centrifugation was repeated and the supernatant once again discarded. The pellet was allowed to air dry for 15 minutes and the pellet was resuspended in sterile distilled water with trituration, and the tube was vortexed. Using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA) RNA samples were quantitated in ng/µl using 2 µl of the RNA extract. The purity (A_{260}/A_{280}) was obtained simultaneously for each sample. After quantitation of the RNA, samples were electrophoresed on agarose gels to check the integrity of the RNA, and then stored at -80°C until cDNA was synthesised.

2.8.4. Agarose gel electrophoresis

Gel electrophoresis is used to separate nucleic acids of different sizes. The nucleic acids are electrophoresed within a gel matrix which is immersed within a buffer solution that provides ions in a constant pH environment to carry the current applied. Because of the phosphate backbone of the molecule, nucleic acids have a consistently negative charge. Therefore, when placed in an electric field they migrate toward the anode. Samples containing nucleic acid are mixed with a loading buffer to weigh them down and loaded into sample wells and an electrical current is applied across the length of the gel. Depending on the concentration of the agarose gel and the voltage applied, different sizes of nucleic acids can be resolved. Visualisation of the nucleic acids bands is facilitated by adding a fluorescent dye to the loading buffer which renders the nucleic acid visible when viewed under ultraviolet light.

Method

Agarose powder was mixed with electrophoresis buffer (1X TAE or 1X TBE, see Appendix 2) to the desired concentration (1-3% weight for volume). The mixture was then heated in the microwave for 30 seconds at a time interspersed with gentle swirling of the flask until the agarose was completely melted. After allowing the solution to cool to 40-60°C, it was poured into a casting tray containing a well-forming sample comb and allowed to set at room temperature. Once the gel had solidified into a slab, the comb was removed and the gel inserted into an electrophoresis chamber, covered with running buffer (1X TAE or 1X TBE, see Appendix 2). Samples containing 8 µl RNA or PCR products were mixed with 2 µl of 5X loading buffer containing both a tracking dye (bromophenol blue) and a fluorescent dye (SYBR gold, Molecular Probes Inc, USA) suspended in glycerol, and were loaded by

pipette into the sample wells. A molecular weight marker (GelPilot 50 bp ladder or GelPilot 100 bp Plus ladder, Qiagen, USA or DNA Molecular Weight Marker VIII (0.019-1.1 kb), Roche Molecular Biochemicals, Germany) was included on the gel for identification of the product size. The power leads were connected and current (80-100V) was applied until the tracking dye had reached two thirds of the length of the gel. The RNA bands or PCR products were viewed and photographed on an ultraviolet lightbox (Spectroline® Transilluminator TS-302, Spectronics Corporation, USA) using a Kodak DC 290 digital camera (Kodak, USA)

2.8.5. Reverse transcription (RT)

Reverse transcription is the process for converting a single-stranded mRNA template to a single-stranded complementary DNA (cDNA). The method works by using a cDNA primer and a reverse transcriptase enzyme, which hybridises to the poly-(A) tail of mRNA in a biological sample, to build a cDNA strand using deoxynucleotides provided in excess in the reaction mixture. This is the preparation step for later exponentially amplifying the first strand cDNA created by reverse transcription through polymerase chain reaction (see 2.8.6.1.). In this way it is possible to detect very small numbers of mRNA molecules within a tissue sample, and thereby obtain a measure of gene expression. In order to protect the stability of the RNA sample and prevent contamination of the specimen, the method used incorporates buffers that eliminate any possible genomic DNA contaminants in the sample, as well as RNAse inhibitors.

Method

Disposable gloves were worn and sterile pipette tips were used at all times. All bench work was carried out on ice. For the reverse transcriptase reaction, 1 µg of RNA was mixed with 1.5 µl gDNA WipeOut buffer (Qiagen, USA) and sterile water to a total volume of 10.5 µl in a 0.6 ml PCR tube. The sample was then placed in the PCR machine (PCR Sprint Thermal Cycler, Thermo Fisher Scientific, USA) at 42°C for 5 minutes after which it was transferred to ice for 5 minutes. Then 1 µl of oligo dT was added to the reaction which was placed in the PCR machine for 5 minutes at 70°C to denature the RNA. It was then transferred to slushy ice for at least 5 minutes to maintain the annealing of the oligo dT and the denatured state of the RNA. Then 8.5 µl of reverse transcriptase mix (ImProm-II™ Reverse Transcription System kit, Promega Corporation, USA) containing 10 mM dNTP, 25 mM $MgCl_2$ and 5X reaction buffer in a volume ratio of 1:2:4 as well as 20 U of RNase inhibitor and 1 µl of reverse transcriptase, was added to the mix containing the RNA sample. For each sample a tube testing for genomic DNA contamination was prepared with all the components except for reverse transcriptase, which was replaced with nuclease-free water. The mixture was pulsed and placed in the PCR machine for 5 minutes at 25°C followed by 1 hour at 42°C to allow the reverse transcriptase reaction to take place for cDNA formation. The reverse transcriptase was then heat inactivated by a 15 minute incubation period at 70°C before using the cDNA samples for polymerase chain reaction (PCR) analysis.

2.8.6. PCR analysis

2.8.6.1. Primer selection

PCR is a primer extension technique for amplifying regions of interest within nucleic acids. The technique relies on the DNA replication ability of thermostable DNA polymerase which uses genomic DNA or cDNA as a template on which it can build a complementary strand. This occurs within an environment of optimum salts, pH and temperature in the presence of an excess of nucleotides from which it builds the new strand. However, DNA polymerase can only start building a new strand from a nucleotide in an existing strand of DNA and thus, a key component for the reaction are the short single-stranded PCR primers designed to hybridise to a specific target sequence on the DNA molecule.

A PCR primer is a synthetically made oligonucleotide of usually 18-24 base pairs designed to prime the DNA template for the attachment of polymerase and direct it to move in a 5' to 3' direction, allowing the DNA template to be duplicated. For PCR, two primers are needed – a forward primer to prime one strand and a reverse primer with a complementary base sequence to prime the complementary DNA strand. The optimal length of 18-24 base pairs is short enough to bind efficiently to the template, but long enough to ensure that the primer is highly sequence specific and unique within the DNA fragment to be amplified. Primers anneal best to the template strand at a temperature that is a function of various factors including the base sequence of the oligonucleotide, percentage GC content, the primer melting temperature, the ionic concentration of the reaction as well as the concentration of primer itself in the reaction.

For the purposes of this study a published primer set (Di Blasio et al, 1997) known to amplify both rat and human was used to amplify the CRF cDNA sequence. The rationale for the use of this primer set was as follows. Since rat gut was to be used as control tissue, sequences with high species homology in the base sequence were required so that common primers could be used for both human and rat tissues. The structure and sequence of rat and human CRF genes are quite similar, both having two exons with an intervening region. The sequences encoding the CRF peptide are highly conserved, showing over 90% homology, with other regions having 70% homology or more. This is illustrated in Figure 2.2. below.

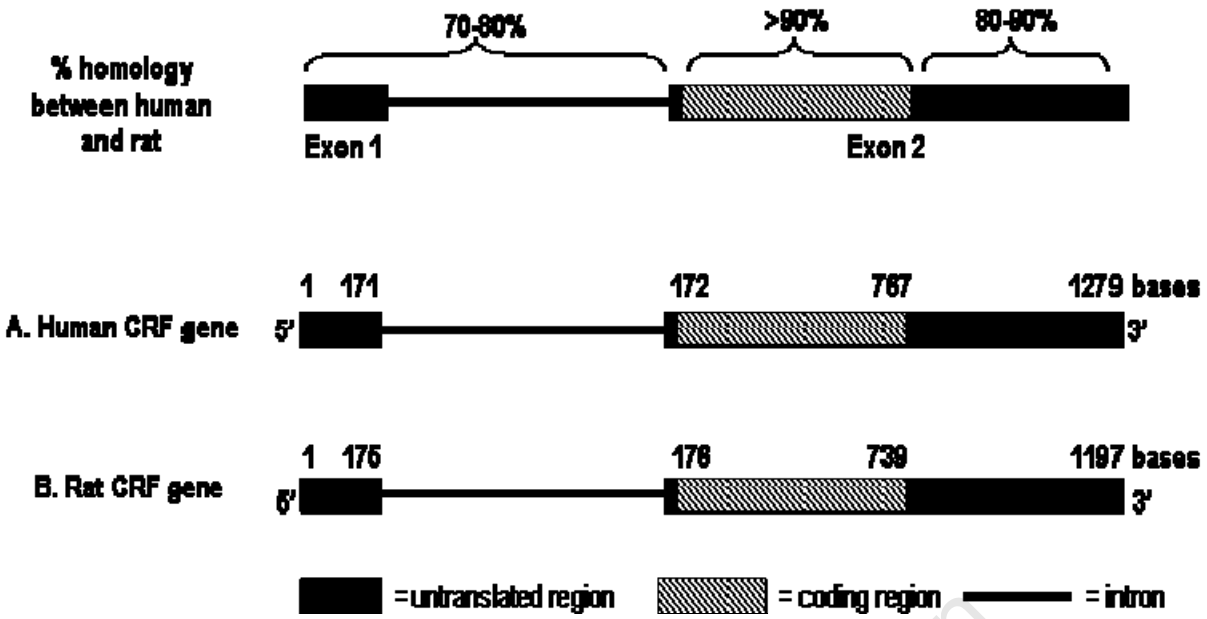


Figure 2.2. Sequence homology and gene structure of human and rat CRF gene

Adapted from Thompson et al, 1987

Ideally, PCR primers for mRNA expression analysis should be designed to include exon/exon junctions. By designing a primer so that part of the primer anneals to the 3' end of one exon and the other part to the 5' end of the adjacent exon, any genomic DNA contamination of RNA samples will not be amplified by PCR, yet cDNA synthesised from mRNA will. In this case, since the CRF coding region is in a single exon, a primer of this design was not possible. Instead, the presence of contaminating genomic DNA was eliminated using gDNA WipeOut buffer in the RT step (see 2.8.5.). Furthermore, the absence of gDNA was confirmed by performing control experiments where reverse transcriptase was omitted from the cDNA synthesis process.

Table 2.3. Sequence alignment for human and rat corticotropin-releasing factor genes

Base	
Human 168	GAAGCGAGTGCCCCTAA - CATGCGGCTGCCGCTGCTTGTGTCCGCGGGAGTCCTGCTGGT GGCTCT
Rat 157	GAAGAGAGCGCCCCTAAACATGCGGCTGCGGCTGCTGGTGTCCGCGGGCATGCTGCTGGT GGCTCT
233	CCTGCCCTGCCCGCCATGCAGGGCGCTCCTGAGCCGCGGGCCGGTCCCGGGAGCTCGGCAGGCGCC
223	GTCGCCCTGTCTGCCT TGCAGGGCCCTGCTGAGCAGGGGAT CCGTCTCTGGAGCGCCGCGGGCCCC
299	GCAGCACCCTCAGCCCTTGGATTTCTTCCAGCCGCCGCCGCAGTCCGAGCAGCCCCAGCAGCCGCAGG
289	GCAGC - - - - - - - - CGTTGAATTTCTTGCAACCG - - - - - - - - - - GAGCAGCCCCAGCA ACCTCAG -
367	CTCGGCCGGTCCTGCTCCGCATGGGAGAGGAGTACTTCCTCCGCCTGGGGAACCTCAACAAGAGCCCGG
335	- - - - - - CCGATT CTGATCCGCATGGGTGAAGA ATACTTCCTCCGCCTGGGGAACCTCAACAGAAGTCCCG
436	CCGCTCCCCTTTCGCCCGCCTCCTCGCTCCTCGCCGGAGGCAGCGGCAGCCGCCCTTCGCCGGAACAGG
400	CTGCTCGGCTGTCCCCCAACTCCACGCCCCTCACCGCGGGTCGCGGCAGCCGCCCCTCGCACGACCAGG
505	CGACCGCCAACTTTTTCCGCGTGTTGCTGCAGCAGCTGCTGCTGCCTCGGCGCTCGCTCGACAGCCCCGC
468	CTGCGGCTAACTTTTTCCGCGTGTTGCTGCAGCAGCTGCAGATGCCTCAGCGCCCGCTCGACAGCAGCAC
575	GGCTCTCGCGGAGCGCGGCGCTAGGAA TGCCCTCGGCGGCCACCAGGAGGCACCGGAGAGAGAAAGG
538	GGAGCTGGCGGAACGCGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAGAGGGAGAGG
642	CGGTCCGAGGAGCCTCCCATCTCCCTGGATCTCACCTTCCACCTCCTCCGGGAAGTCTTGGAAATGGCCAG
605	CGGTCCGAGGAGCCGCCCATCTCTCTGGATCTCACCTTCCACCTTCTGAGGGAAGTCTTGGAAATGGCCAG
713	GGCCGAGCAGTTAGCACAGCAAGCTCACAGCAACAGGAAACTCATGGAGATTATTGGGAAATAAAACGGT
676	GGCAGAGCAGTTAGCTCAGCAAGCTCACAGCAACAGGAAACTGATGGAGATTATCGGGAAATGAAATGTT
783	GCGTTTGGCCAAAAAGAATCTGCATTTAGCACAAAA - - - - - - AAAATTTAAAAAAATACAGTATTCTGTACC
746	GCGCTTGGCCAAAACGATTCTGCATTTAGCACACAAGTAAAAATAAAAAATTTAAAACACAGTATTCTGTACC
849	ATAGCGCTGCTCTTATGCCATTTGTTTATTTTTATATAGCTTGAAACATAGAGGGAG - AGAGGGAGAGAGCC
820	ATACTGCAGCTCTGATATCATTTGTTTATTTTTATATAGCTTGAAGCATAGAA GATGT ACAGGGAGAGAGCC
920	- - TATACCCCTTACTTAGCATGCACAAAGTGTATTCACGTGCAGCAGCAACACAATGTTATTCGTTTTGTCT
991	TATATACCCCTTAATTAGCATGCACAAAGTGTGTTTCTT TGTAGTA ACAAAACAGCGTTATTTGTATTGTTC
990	ACGTTTAGTTTCCGTTTCCA - - - - - - GGTGTTTATAGTGGTGTTTTAAAGAGAATGTAGA -CCTGTGAGAAAA
963	ACGCTTAGTTTCTATGTGCAAATAAGTGTCTTTATAGCGATATCTTAAAGAAAATGTGGATCCAAGGAGGAAA
1056	CGTTTTGTTTGAAAAAGCAGACAGAAGTCACTCAATTGTTTTT - GTTGTGGTCTGAGCCAAAGAGAATGCCA
1036	C - - - - - CTTTAAAAAAGCAGATGGAAGTCACCCAGTTGTTTTTAT TTGGAGACACAGTGTAAGAGAATT CAT
1127	TTCTCTTGGGTGGGTAAGACTAAATCTGTAAGCTCTTTGAAACAACTTTCTCTTGTAAACGTTTCAGTAATAA
1103	TCTTGAGGGGTGGCTAGGACAAAATGTGTAAGCTCTTTGAATCAACTTTTTCTTGTAAATGTTTCAATAATAA
1200	AACATCTTTCCAGTCCTTGGTC
1176	AACATCTTTCTGATCCTTGGTC

Key:

Exon boundary

Forward primer

Reverse primer

2.8.6.2 PCR

The PCR reaction containing the primers, polymerase enzyme and deoxynucleotides is sequentially heated and cooled in a number of cycles during which millions of copies of the DNA fragment of interest are generated. This occurs because at high temperatures the double helix of the double-stranded DNA becomes denatured and separates into two strands. Cooling then enables the primers

to anneal to the single-stranded DNA and serve as starting points for DNA polymerase to synthesise a new strand homologous to the cDNA template. Subsequent higher temperatures allow extension of the new DNA using free deoxynucleotides available in the reaction mix. The three steps of denaturation, annealing and extension are repeated in 25-45 cycles, resulting in the accumulation of the desired product.

Method

A GeneAmps PCR System 2700 (Applied Biosystems, USA) PCR machine was used to amplify the CRF fragment using the cDNA generated from the first strand synthesis reaction described above. The PCR reaction used a total volume of 7.5 µl as follows using 0.5X FailSafe™ buffer (Epicentre Biotechnologies, USA), 1 µl each of cDNA template, 0.67pmol of each forward primer and reverse primer (each at a concentration of 2.5 pmol), and 0.5 units Taq (*Thermus aquaticus*) polymerase (Bioline, USA). The remaining volume was made up with sterile water. The forward primer sequence used was 5' – TTTCCGCGTGTTGCTGC -3' and the reverse primer sequence used was 5' – TTCCTGTTGCTGTGAGC-3' (Table 2.3.). CRF primers were synthesised by IDT (Belgium) and supplied by Whitehead Scientific Pty Ltd (SA). For the amplification of the housekeeping genes, glyceraldehydes dehydrogenase (GAPDH) and glucuronidase (GUS1B), commercial primer sets were used (QuantiTect® Primer Assays, Qiagen, USA).

At the start of the study, a 3 step PCR protocol after Di Blasio et al (1997) which included a single initiation cycle of 95°C for 4 minutes, 56°C for 2 minutes, and 72°C for 2 minutes, followed by 35 amplification cycles of 95°C for 40 seconds, 56°C for 30 seconds and 72°C for 1 minute, followed by a final extension step of 72°C for 15 minutes, was used. Because this was not optimal, these conditions were gradually optimized based on the results (see Results). The optimising process included the use of gradient PCR (Multigene^{TM} Gradient Thermal cycler, Labnet International Inc, Korea) to identify the most favourable temperatures for the cycle conditions. Fully optimized PCR conditions, which yielded a single PCR product of the expected size of 233 bp without concurrent production of non-specific products or primer dimers were as described by Di Blasio et al (1997), but with changes to the annealing temperature, which was 50-52^{o}C**.**

2.8.6.3. Restriction enzyme digestion

This method employs restriction endonuclease enzymes to cleave DNA strands at specific recognition sites of about 4-8 base pairs on the molecule. By selecting an enzyme that has a known recognition site within the fragment amplified by PCR it is possible to confirm that the PCR product is the desired product by performing restriction digestion. By incubating the purified PCR product with the restriction enzyme and an enzyme-specific buffer under optimal conditions of salt concentration, pH and temperature, the enzyme will cleave the DNA molecule in a predictable manner producing digestion fragments of a known size. The digested fragments can then be separated and visualised by gel

electrophoresis and the sizes estimated with the aid of a DNA molecular weight marker. The presence of fragments of the predicted sizes confirms that amplification of the correct target sequence had occurred in the PCR. From Di Blasio et al (1997) it was known that there is a PstI recognition sequence in both rat and human CRF. The correct size of the digestion products was ascertained by searching the sequence between the primers for PstI sites using Webcutter 2 (http://rna.lundberg.gu.se/cutter2/). For the rat CRF sequence, PstI recognised two sites near each other, which would cleave the PCR product into 3 fragments of 207 bp, 18 bp and 9 bp. The human CRF sequence has a single PstI cleavage site which yields digestion products of 215 bp and 18 bp. This is shown graphically in Figure 2.2 below.

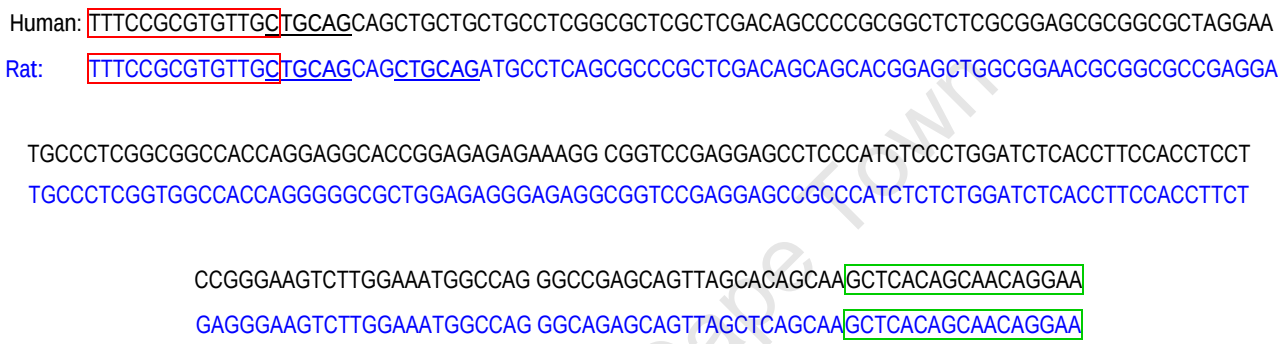


Figure 2.3. Nucleotide sequence for CRF indicating PstI recognition sites

Key:

Forward primers

Reverse primers

PstI recognition sequence (Cleavage: ctgca/g)

Method

After PCR amplification and agarose gel electrophoresis, the correctly sized PCR product was excised from the gel and cleaned using Wizard SV Gel and PCR Clean Up System (Promega Corporation, USA) to remove excess nucleotides and primers according to the kit protocol. This involved melting the weighed gel piece in an equal volume of guanidine isothiocyanate solution (Promega Membrane Binding Solution) in a sterile microcentrifuge tube with vortexing and incubation at 65°C for 10 minutes. The dissolved gel mixture was then transferred to a SV minicolumn which had been placed in a collection tube and was incubated for 1 minute at room temperature. The minicolumn assembly was then centrifuged at 14000 rpm for 1 minute at room temperature and the liquid in the collection tube was discarded. The minicolumn was washed with 700 µl of Promega Membrane Wash solution previously diluted with 95% ethanol and the 14000 rpm centrifugation was repeated for 1 minute. The wash was repeated using 500 µl of Promega Membrane Wash with centrifugation at 14000 rpm for 5 minutes. The collection tube was once again emptied and the

minicolumn assembly was centrifuged for a further 1 min at 14000 rpm with the lid off to allow for evaporation of any residual ethanol. The purified DNA was finally eluted into 20-30 µl of nuclease-free water by centrifugation for 1 minute at 14000 rpm.

For restriction enzyme digestion 10 µl of the purified PCR product DNA was added to 2 µl of 10X SuRE/Cut buffer H (Roche Molecular Biochemicals, Germany),and 6 µl sterile water. Digestion was effected by incubating the mixture with 1 µl (10 U) of PstI (Roche Molecular Biochemicals, Germany) for 1 hour at 37°C followed by an overnight incubation at room temperature with an additional 10 U of PstI. The digestion products were resolved by electrophoresis on a 3% agarose gel against a molecular weight marker.

2.8.6.4. Cycle sequencing

Cycle sequencing is a technique used to determine each base and the sequence in which the bases occur in a fragment of DNA. Like PCR, cycle sequencing requires a primer, polymerase enzyme and dNTPs to synthesise new DNA product in a number of thermal cycles. However, the intention is to linearly rather than exponentially amplify the DNA fragment of interest and therefore it is possible to use a primer in one direction only. Cycle sequencing includes dideoxynucleotides which, when randomly incorporated into the new DNA sequence, are able to terminate the extension process. In dye termination sequencing, each of the different dideoxynucleotides (adenine, cytosine, guanine and thymine) incorporates a fluorescent dye of a different colour. Therefore, where DNA products of differing lengths have been generated, it is possible to identify the last base of each. Ideally, a fragment will be generated which represents each base in the original DNA template of interest. Once the cycle sequence reaction has been completed, the sample is analysed using a DNA sequencer, which separates the DNA fragments by electrophoresis. A laser illuminates the fluorescent dyes on the DNA fragments and a camera is used to detect the coloured light emitted. The sequence of bases can then be read.

Method

PCR products were excised from the agarose gel and cleaned in the same manner as prior to restriction enzyme digestion (see section 2.8.6.2.). Purified PCR products were then sequenced in the forward direction. Using the same oligonucleotides used for the original PCR reaction, cycle sequencing reactions were performed using the BigDye® terminator cycle version 3.1 sequencing kit (Applied Biosystems, USA). Briefly, a final reaction volume of 20 µl comprised 2-5 µl of PCR product, 3 pmol of the sense primer, 1x BigDye reaction buffer and 1x termination mix. The cycling conditions used were 96°C for 5 minutes followed by 25 cycles of 95°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes. Thereafter, the sequencing reactions were cleaned by ethanol precipitation and resuspended in 10 µl water. Then 5 µl of the sequenced products were mixed with 7 µl Hi-Di ™ formamide (Applied Biosystems, USA) and were heat denatured prior to electrophoresis on an ABI

Prism™ 3100 automated sequencer (Applied Biosystems, USA). Analysis was performed using the ABI Prism™ DNA Sequencing Analysis Software version 3.7. The identity of the sequence was determined by entering the sequence into NCBI basic local alignment search tool (BLAST) (www.ncbi.nlh.nih.gov) and checking for homology to the known sequence for rat of human CRF.

2.8.7. Tissue histology

Method

The tissue specimen collected during the surgical procedure was divided with a clean scalpel blade. The full thickness small intestine section (approximately 0.5cm³ in size) was placed mucosa side down onto clean filter paper and immediately placed into cold 4% paraformaldehyde for fixing. Once fixed for 24 hours it was transferred to 70% ethanol and refridgerated until it was processed over a 24 hour cycle through 10% buffered formalin, 70% to absolute alcohol, xylol and finally to paraffin wax embedding.

Haematoxylin and Eosin (H&E) staining

Four micron embedded sections were dewaxed in xylol and hydrated to water. They were placed in Mayer's Haemalum for 5 minutes and the excess dye was rinsed off with water. Sections were then differentiated in 1% acid alcohol followed by a rinse under running tap water for 1 minute. Thereafter the sections were allowed to blue in Scott's Tap Water Substitute for 1 minute and were again rinsed under running tap water for 1 minute. Sections were then stained with Eosin/Phloxine for 2 minutes, briefly washed to remove excess stain and were drained. The mounted sections were then dehydrated through graded alcohols, cleared in xylol and coverslipped.

Bismarck Brown staining

Four micron sections were taken to 70% alcohol, then placed in filtered Bismarck Brown solution for 30-90 minutes. Sections were then rinsed in 70% alcohol and counterstained in haematoxylin for 1 minute. This was followed by blueing in tap water and rinsing in distilled water. They were differentiated for 2 seconds each in 3 changes of 70% alcohol. Sections were then quickly dehydrated in 96% through absolute alcohol, cleared in xylol and mounted in Entellan® Rapid Embedding Agent (Electron Microscopy Sciences, USA).

2.8.8. Quantification of mast cells

Method

Using a calibrated graticule, the view frame of the microscope was measured in both the vertical and horizontal planes and the area of the field demarcation calculated. This area became the counting frame. Using the 10x magnification lens and brightfield imaging, each slide was examined and the mast cells counted as follows. Examining each tissue zone/layer of the specimen separately i.e. mucosa, submucosa, muscularis and serosa, the total number of mast cells was counted for each

calibrated counting frame field. Noting histological landmarks, sequential fields were similarly counted for each zone so that the entire section (100% of slide) was eventually examined. No fields were excluded. All cells stained with Bismarck Brown stain were counted as mast cells. Mast cells were counted if they fell entirely within the calibrated frame of the view field or if more than 50% of the cell was within the counting frame but touched the border of the frame.

The mean number of mast cells for each tissue layer was calculated by dividing the sum of the total number of mast cells per field by the number of counting fields comprising the tissue layer in question. Finally, the number of mast cells per mm^2 was calculated based on the known area of the counting frame.

2.9. Statistical Analysis

2.9.1. Sample size calculation

Setting the alpha error at 0.05 and the beta error at 0.2, the sample size required for statistical power to detect a 20% inter-group difference was found to be 7 patients per group, assuming the standard deviation was equal for both groups.

2.9.2. Statistical analysis

Data skewness was tested using the Shapiro-Wilks test. Much of the data was skewed. Descriptive statistics were applied to patients' demographic and clinical data. Skewed data was expressed as median (interquartile range) while data fitting the normal distribution was expressed as mean (95% confidence interval). Between group differences for normal data were tested using the independent T-test or the Mann-Whitney U test in the case of non-parametric variables. The CRF levels at the different time-points and change from baseline within and between groups was tested by repeated measures ANOVA. Two-way factorial ANOVA was used to measure inter-group differences in physiological tests and clinical indicators. For significant ANOVA models, post-hoc testing was done using Tukey's Honest Significant Difference Test. Secondary analysis included correlations using Spearman's Correlation. Statistical significance was taken as a p-value of <0.05 in all cases. Results with a p-value of <0.15 were considered to be trending toward statistical significance. Comparisons of data from this study and published data were performed using the "comparison of means" test of the significance testing module of the statistical software programme. Analysis was performed using Statistica 6.0 (Statsoft, USA).

Chapter 3: Results

3.1. Patients

3.1.1. Patient characteristics

Recruitment for the study took place between May 2005 and February 2007. For trauma patients, recruitment relied on a system of referrals of potential patients by the Trauma Unit staff or the trauma surgeon on call. The principle investigator was constantly on call to the Trauma Unit to receive referrals during this time. Elective patients were screened and recruited via the case booking list of the Hepato-biliary Firm of the academic hospital. During the study period a total of 100 patients were screened for possible inclusion into the study. Of these, 25 patients could be included in the study: - 8 trauma patients and 17 elective surgery patients. Table 3.1. summarises the demographic data and baseline characteristics of the 2 patient groups. The trauma group consisted entirely of patients with gunshot wounds involving the abdomen while the elective group mainly had bile duct or pancreatic lesions, which required surgical intervention.

Predictably, the trauma group were statistically younger and included proportionately more males. Groups were matched for the prognostic Acute Physiology and Chronic Health evaluation (APACHE) II score (see Appendix 3). However, it should be noted that the APACHE II was calculated from clinical data from the first 24 hours in ICU. As with this study group, trauma patients worldwide tend to be young. They also generally score low on the APACHE after resuscitation and surgery, because the scoring system loads chronic disease and advancing age in order to prognosticate. However, pre-resuscitation APACHE scores for patients with severe traumatic injury can be high because of markedly deranged acute physiological variables. Once stabilised and in ICU, APACHE scores are once again reduced. Therefore, while the study groups were matched for APACHE, this matching probably reflects an age difference and does not accurately represent how systemically stressed the trauma group were when compared with the stable elective patients. Severity of injury in the trauma group was also evaluated using the Injury Severity Score (see Appendix 3) for which there is no comparator among stable surgical patients, and the need for mechanical ventilation. All trauma patients underwent a laparotomy with repair of internal injuries, which included resection of damaged small bowel with primary anastomosis. Elective surgery patients all underwent some form of intestinal bypass procedure which involved removal of small sections of normal small bowel in order to create the enteroanastomosis. Trauma patients reached hospital within a mean of 3 hours and reached surgery within a mean of 6.5 hours following their injury. All trauma patients were shocked upon arrival, having a mean (95% confidence interval) lowest recorded systolic blood pressure of 76.1 (54-99) mmHg. This group also required significantly more intravenous fluids in the first 24 hours following injury, and received double the fluid volume of the elective group. The mean duration of surgery was slightly over 3 hours in trauma patients, and less than 4.5 hours in the elective patients. The group difference in surgery duration trended towards being longer in the elective patient group.

Table 3.1. Demographic and baseline characteristics

	Trauma group (n=8)	Elective group (n=17)	p-value
Age (years)	28.1 (20-36)	50 (45-55)	<0.0001
Gender distribution M/F	8/0	10/7	0.032
Diagnosis	Penetrating abdominal trauma (8 pts)	Bile duct obstruction/ injury (4 pts) Pancreatic lesion (8 pts) Chronic calcific pancreatitis (5 pts)	
Surgical procedure	Laparotomy with various repairs of internal injuries, including small intestinal resection and primary anastomosis (8 pts) *2 patients also underwent subxiphoid window which progressed to sternotomy in 1 pt*	Hepaticojejunostomy and enteroanastomosis (7 pts) Whipple's procedure (4 pts) Gastrojejunostomy and enteroanastomosis (3 pts) Choledocotomy, cholecystojejunostomy/ choledocojejunostomy and Roux loop enteroanastomosis (2 pts) Pancreaticojejunostomy and enteroanastomosis (1 pt)	
APACHE II score (first 24 hours in ICU)	7.5 (2.8-12)	7.25 (4.6-9.9)	0.9
Injury Severity Score (ISS)	26.5 (20-37)		
Patients receiving mechanical ventilation (Y/N)	4/4	1/16	0.01
Time between injury and hospital arrival (min)	183 (84-282)		
Time between injury and surgery (min)	388 (260-516)		
Minimum baseline systolic blood pressure (mmHg)	76.1 (54-99)		
IV fluids administered in first 24 hours (ml)	11450 (8332 – 14567)	5479 (4532-6436)	0.00001
Duration of surgery (min)	189 (159-220)	266 (206-325)	0.08
Total morphine via epidural route in first 24 hours (mg)	No patients on epidural morphine	3 (0-7.2)*	
Total morphine dosage in first 24 hours (mg) (combined epidural, intravenous and intramuscular routes)	40 (24.5-53)*	10 (6-30.5)*	0.014

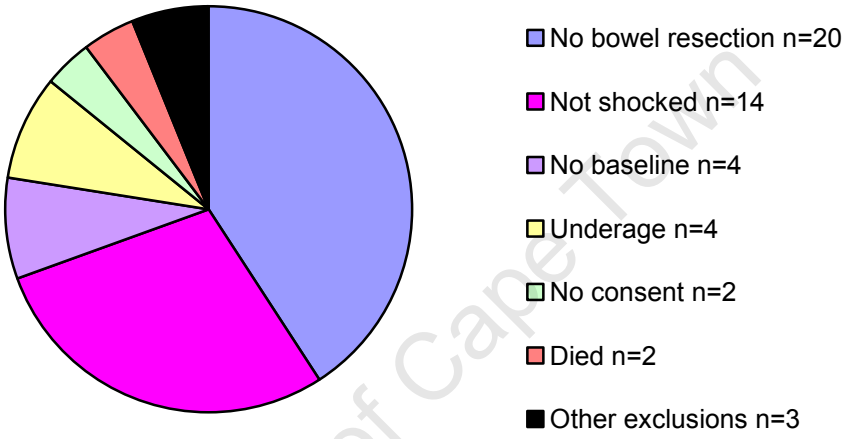
APACHE II: Acute Physiology and Chronic Health Evaluation

All data: mean (95% confidence interval) except *median (IQR)

3.1.2. Screen failures

Of the 100 patients screened, 75 were excluded. Additionally, an unknown but substantial number of trauma patients could not be included because they were not timeously referred to the study investigator during the pre-operative period. Figures 3.1.A and 3.1.B. indicate the reasons for exclusion in the 2 patient groups. In the trauma group, the main reasons for screen failure were no bowel injury at laparotomy and the absence of the pre-defined indicators of shock. The main reason for screen failure in the elective group was that patients declined to participate in the study.

A. Trauma group n=49



B. Elective group n=26

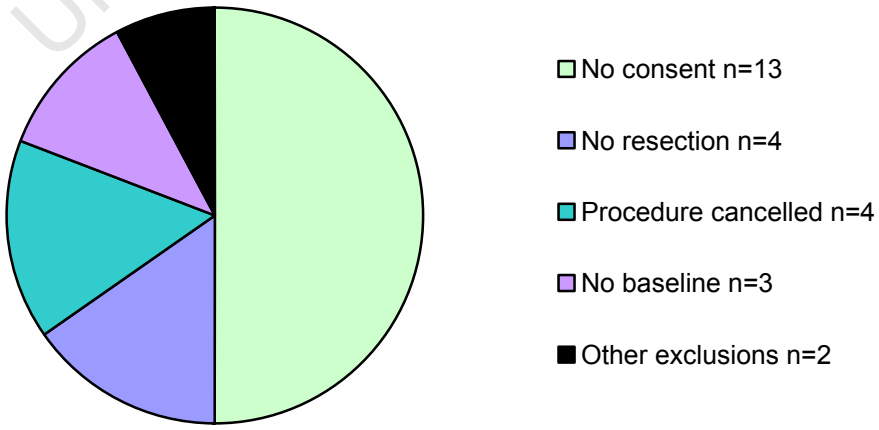


Figure 3.1. Screen failures

The outcome variables for the 2 groups are summarised in Table 3.2. below. The trauma patients required significantly more days on ventilatory support compared with the elective patients. This group underwent statistically more re-laparotomy procedures, since no re-operations were performed among the elective group. The trauma group also showed a trend toward a longer hospital stay, more nosocomial infections and higher mortality. The trauma group had a median predicted risk of mortality of 15%, which is derived from the Injury severity Score (Champion et al, 1989). One trauma patient died while in hospital of a suspected pulmonary embolism after being discharged from the ICU.

Table 3.2. Outcome variables

	Trauma group (n=8)	Elective group (n=17)	p-value
ICU days	3 (0.4-5.6)	1.9 (1.2-2.5)	0.2
Ventilator days	1.37 (-0.2-2.9)	0.06 (-0.7-0.18)	0.007
Number of re-operations	0.62 (-0.4-1.6)	0 (-)	0.04
Number of nosocomial infections	1.25 (0.4-2.1)	0.5 (0.1-0.9)	0.052
Hospital days	17.6 (7-28)	12.8 (10-15)	0.15
Predicted risk of mortality (%) based on Injury Severity Score	15 (6-41)*		
Hospital mortality – Lived/Died	7/1	17/0	0.14

Mean (95% CI) * median (IQR)

In the trauma group ISS was associated with days on ventilation (Spearman's R = 0.86, $p<0.05$). APACHE score was positively correlated with length of ICU stay (Spearman's R = 0.87, $p=0.005$).

3.2. CRF concentrations

3.2.1. Plasma CRF

The first objective was to measure and compare corticotropin-releasing factor (CRF) concentrations in the peripheral circulation of patients in the two study groups. The blood samples taken at baseline (pre-operatively), intra-operatively and after the first 24 hours post-operatively were analysed for CRF peptide concentration using radio-immunoassay (RIA).

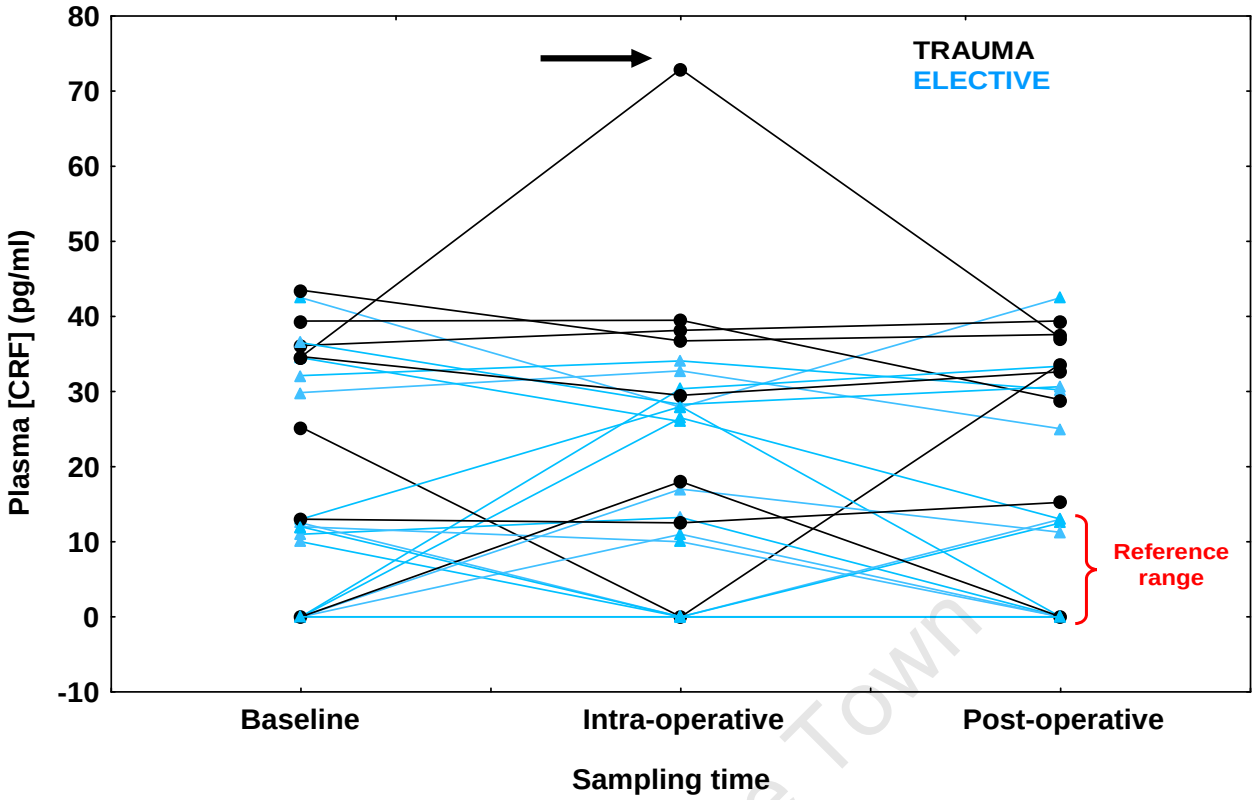


Figure 3.2. Case profiles for plasma CRF for individual patients

Figure 3.2. depicts case profiles for plasma CRF levels for each individual patient in the 2 groups. The individual trauma patients are represented by solid dots and lines in black ink and the elective group by solid triangles and dotted lines in blue. Baseline plasma levels of the CRF peptide were undetectable in 5 elective patients, and 1 trauma patient. Intra-operative levels were below the detection limits in 5 elective and 1 trauma patients. In the samples taken at 24 hours post-operatively, CRF concentrations were undetectable in 7 elective patients as well as 1 trauma patient.

It is apparent from the graph that one patient in the trauma group is a clear outlier at the intra-operative sampling time (see arrow). The explanation for this data point being double that of the group median is that the duplicate samples assayed for this individual were widely disparate, with one exceptionally high gamma count and one relatively low count. Since CRF concentrations derived from duplicate samples are averaged, this data point appears much higher than others in the same study group at this time point - it falls outside the group inter-quartile range. Since it is impossible to be certain which of the duplicate results indicates the true CRF value for this patient, the outlier was excluded from group analysis of the data. By the same logic the data point for another patient in the trauma group at the intra-operative sample time was also excluded as an outlier.

The group median (IQR) values for plasma CRF are shown in Table 3.3. A repeated measures ANOVA to test for a difference between the 3 sampling times revealed no statistical difference between median values at baseline, intra-operatively or 24 hours post-operatively within each group.

However, the group interaction effect was significant (p=0.04) in this ANOVA model, indicating that inter-group differences exist. Post-hoc testing confirmed inter-group differences at all sampling times. In each case, elective patients had significantly lower plasma CRF concentrations, which were statistically significant compared with those of the trauma patients. There was no difference between the plasma CRF levels between male and female elective patients at any time point.

Table 3.3. Group median (IQR) plasma [CRF] (pg/ml)

Group	Baseline (pre-operatively)	Intra-operatively	24 hours post-operatively	Intra-group p-value (ANOVA)
Elective (n=8)	12 (0-29.8)	17 (0-27.9)	11.9 (0-27.6)	0.57
Trauma (n=17)	34.6 (19.1-37.7)	29.5 (12-38.8)	33.1 (22 –37.4)	0.79
Inter-group p-value (ANOVA)	**0.007**	**0.022**	**0.03**	

Reference range 0-10 pg/ml, as provided by kit manufacturer

The results therefore indicate significantly higher circulating CRF levels at all time points among trauma patients. Interestingly, median values for this group were three times the concentration currently considered to be the upper end of the reference range for plasma CRF, with the lower quartile also being above the maximum reference concentration. While median values for the elective group also exceed the known upper reference value, concentrations were still less than half of the elevated concentrations seen in the trauma group. Additionally several elective patients had CRF concentrations considered “normal”, given the reference range used. This was not the case among the trauma group.

Presumably this is related to the different degree of stress experienced by trauma patients compared with elective patients. While elective patients generally do experience anxiety prior to undergoing surgery, the extent of physiological stress is minimal compared to a patient with acute traumatic injury. In the trauma group the Injury Severity Score was positively associated with 24 hour post-operative plasma CRF concentration (Spearman’s R = 0.74, p=0.09). This is an expected correlation between degree of physical injury and the integrated stress-response of which plasma CRF is a marker. In this group a sustained high plasma CRF level is seen throughout the first 24 hours following injury, with the 24 hour CRF concentration being significantly and strongly correlated with injury severity.

In the elective group the induction of anaesthetic (together with surgery) is in itself a known inducer of stress hormones. In fact this is the major physical stressor in elective patients who are relatively unstressed by comparison. The increase, albeit not statistically significant, in plasma CRF during the

intra-operative period compared with other sampling points reflects this. The return to baseline levels at 24 hours post-operatively presumably is an indication that the stress imposed by anaesthetic and surgery is a transient effect in the absence of severe traumatic injury. Additionally, ten patients in this group received epidural analgesia started during the operative procedure, which itself is known to reduce physiological stress (Kehlet and Moesgaard, 1996).

3.2.2. Tissue CRF

Having found a group difference in plasma CRF levels, the next experiment and the second objective of the study was to measure and compare CRF peptide levels in intestinal tissue from both trauma and elective patients. This was achieved by extracting the peptide from small bowel tissue and quantitating CRF levels using radio-immunoassay (RIA). Final tissue CRF levels were referenced against total protein content of the tissue extract. In this way, the final tissue CRF levels accounted for differing specimen sizes, tissue total protein content and extent of bowel oedema.

The median (IQR) total protein content of the intestinal tissue specimens was 0.48 (0.4-0.9) µg of protein per µg tissue in the elective surgery group versus 0.74 (0.5-0.9) µg protein per µg tissue in the trauma group. This difference was not statistically significant (p=0.32). The CRF levels measured in intestinal tissue were then expressed as a percentage of the total protein. These median (IQR) values were 0.0049 (0.0039-0.0065) % of total protein for elective patients and 0.0043 (0.0032-0.0054) % of total protein for trauma patients (Figure 3.3.). No inter-group statistical difference was found (p=0.5).

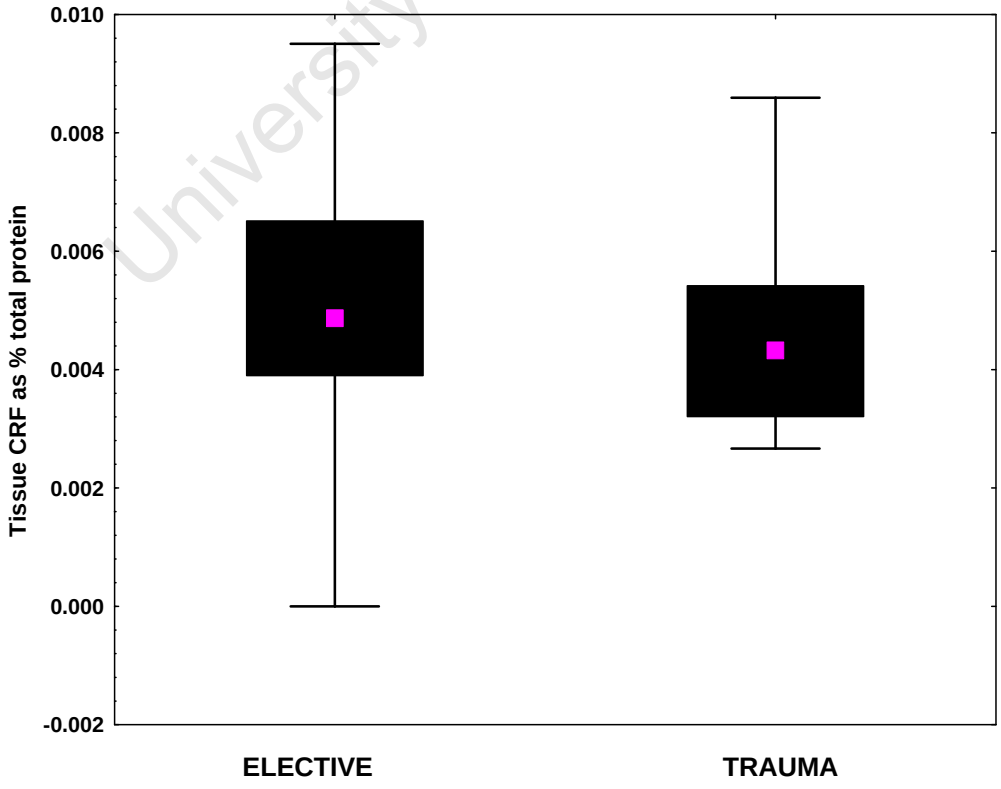


Figure 3.3. Median tissue CRF levels expressed per total protein

In the trauma group the tissue CRF levels correlated with circulating plasma CRF levels taken at the same time point during surgery (Figure 3.4.). The association was strongly positive and was statistically significant (Spearman's R = 0.73, p = 0.03). In the elective group such a correlation was not found.

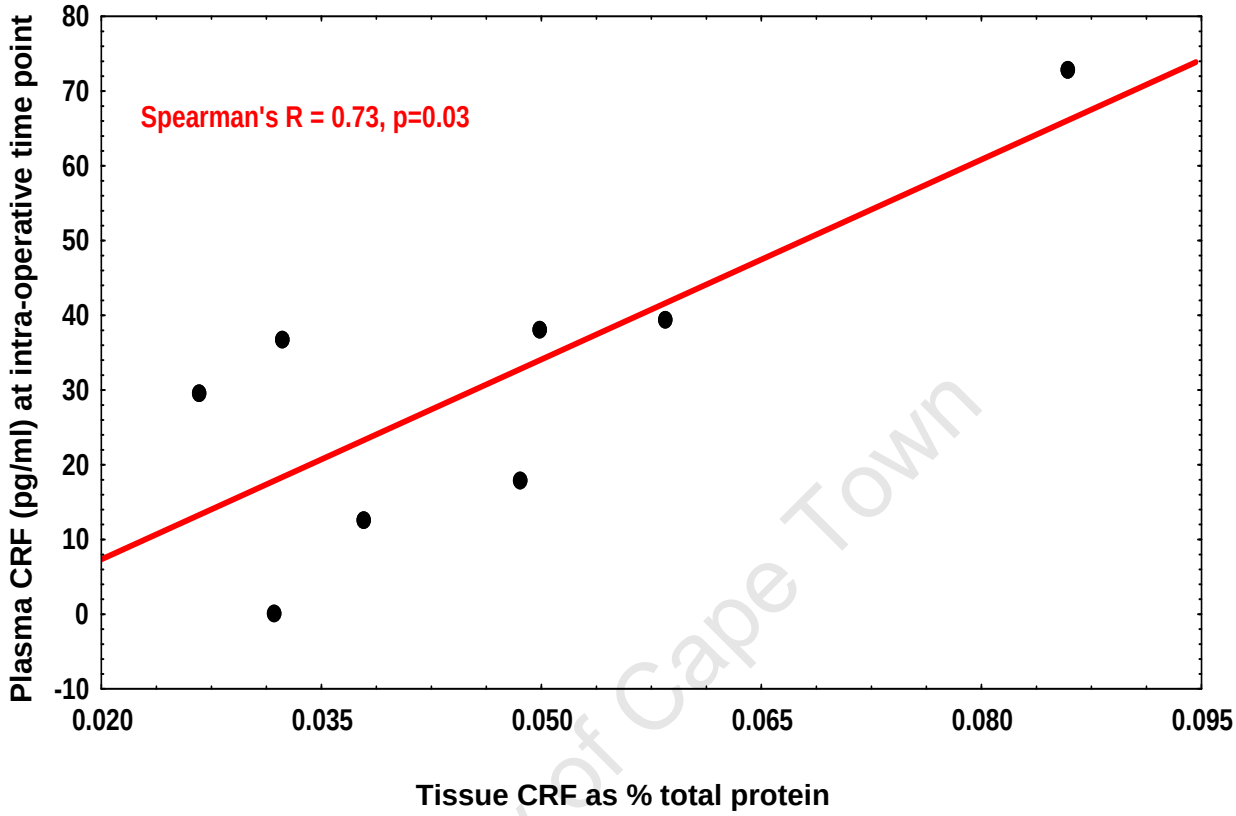


Figure 3.4. Tissue CRF vs plasma CRF in trauma cases (n=8)

3.3. Expression of CRF mRNA

The measurement of CRF peptide in intestinal tissue extracts does not enable one to distinguish between centrally synthesised CRF and that synthesised in tissue. Therefore, to determine directly whether CRF is synthesised in gut tissues, the next objective was to measure CRF mRNA in tissue specimens. The approach taken was to extract RNA from homogenised tissue samples and use RT-PCR to detect the CRF mRNA transcripts.

3.3.1. Expression of CRF mRNA in rat tissues

Rat brain, rat hypothalamus and rat gut tissue were used as positive controls for the purposes of optimising the RT-PCR conditions because CRF is widely expressed widely in these tissues. Given the expected challenges of working with human intestinal tissues, storage and homogenisation methods were tested on rat gut.

The RT-PCR technique relies heavily on purification of a good quality mRNA template which is checked by gel electrophoresis and quantitated spectrophotometrically. RNA extracted from all three rat tissues revealed clear, sharp 28S and 21S bands, indicating that the RNA was of good quality and not degraded.

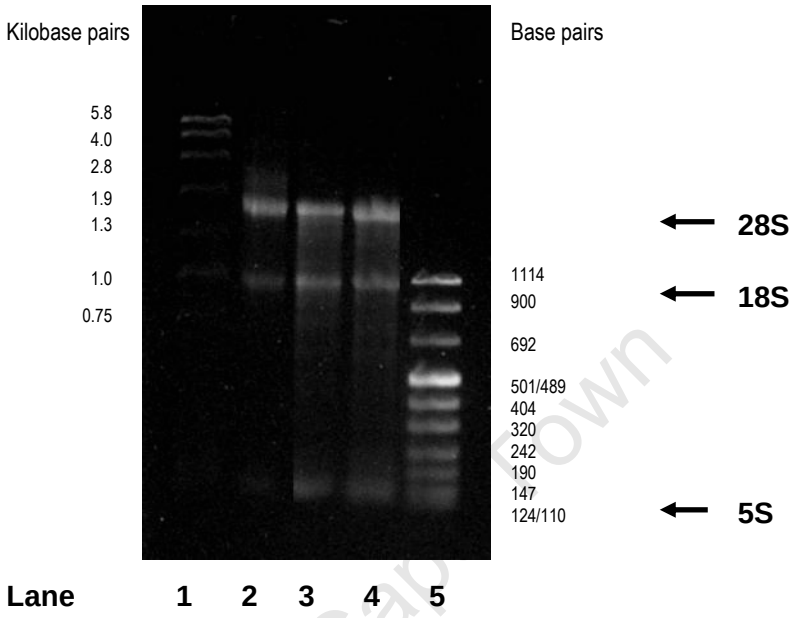


Figure 3.5. Electrophoresis of RNA from rat tissues

Lane 1 and 5: molecular weight marker; Lane 2: hypothalamus; Lane 3: brain; Lane 4: gut

This RNA was used to synthesise cDNA using gDNA wipe-out buffer or treating the RNA with DNAse 1 to eliminate genomic DNA which may have contaminated the mRNA sample. This was done because, as was noted earlier, (see section 2.8.6.1.) it was not possible to design primers that could distinguish between cDNA and gDNA, because the coding region is a single exon. The cDNA was then used as a template in PCR to amplify the CRF transcripts with a primer set that amplifies both rat and human CRF. The PCR cycling conditions as published by Di Blasio et al (1997) were used.

The expected size of the CRF fragment was 233 base pairs. Initially, using the FailSafe™ buffer (routinely used for high GC content DNA targets), amplification products ranged from 300 – 500 bp with either a single incorrectly sized fragment with non-specific amplicons (at 56^{o}C), or a fragment of the correct size with an additional product of about 600 bp (at 58^{o}C) (Figure 3.6.A and B respectively).

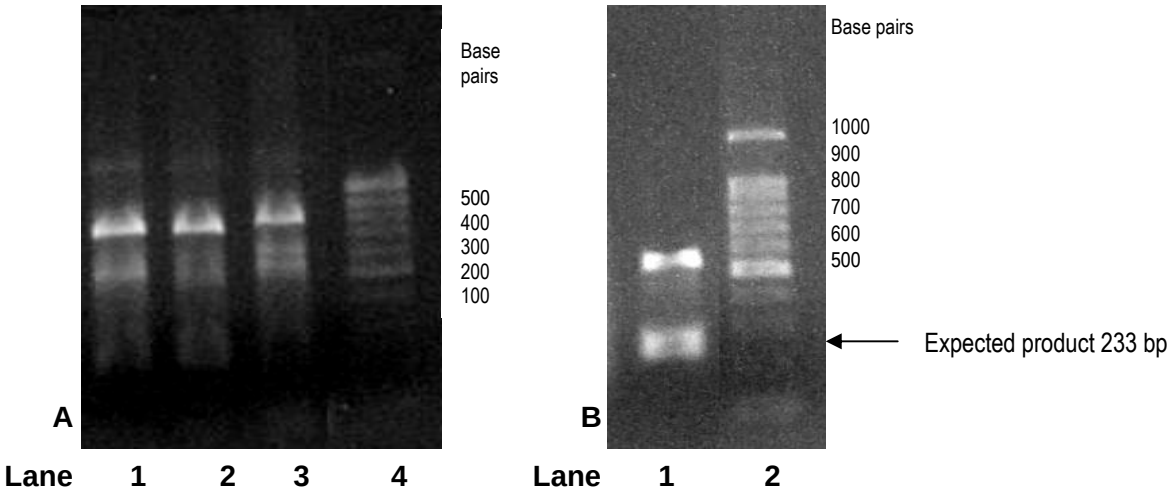


Figure 3.6. RT- PCR products from rat RNA amplified at different annealing temperatures:

3.6.A) at 56°C a product of about 300bp with other non-specific products were observed.

Lane 1: rat brain, Lane 2: Rat hypothalamus, Lane 3: rat gut, Lane 4: molecular weight marker;

3.6.B) at 58°C a product of about 233bp with an additional fragment of > 500bp were resolved.

Lane 1: Rat hypothalamus, Lane 2: molecular weight marker

Optimization attempts to amplify the correct product included using different PCR buffers and their respective Taq polymerases (Bioline and Supertherm), increasing magnesium concentrations from 1.5mM-3mM, and increasing annealing temperature from 56°C-62°C. Increasing the annealing temperature to 58°C during the initiation step and 60°C during the amplification cycles with FailSafe™ buffer and 1.5mM MgCl resulted in a correctly sized single fragment being amplified for rat brain, hypothalamus and gut tissue. Although these analyses are not quantitative, the product of the rat gut was considerably fainter than the products for the other tissues (Figure 3.7.). This is most likely due to the lower percentage copy number of CRF mRNA in the total gut RNA extract.

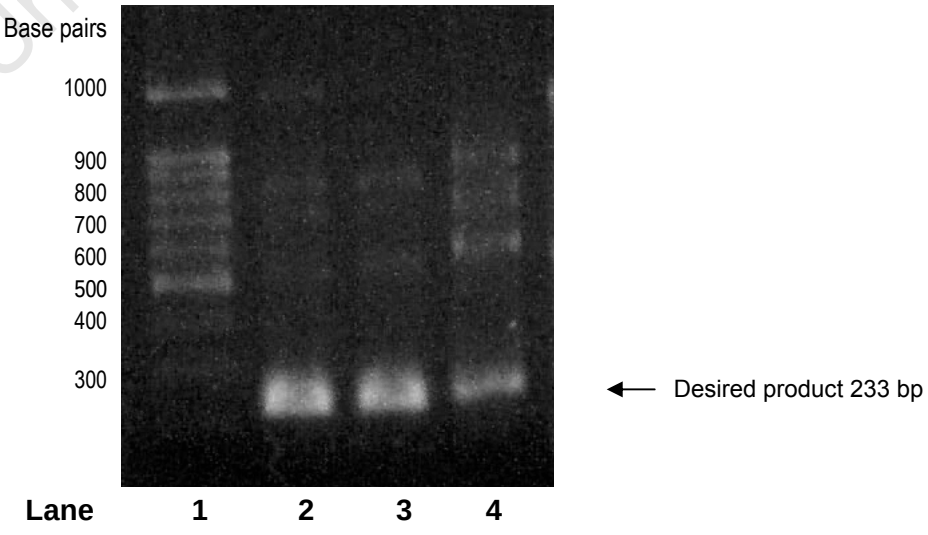


Figure 3.7. CRF RT-PCR products from rat brain (lane 2**), hypothalamus (**lane 3**) and gut (**lane 4**)**

Lane 1: molecular weight marker

The presence of a few non-specific bands in the PCR necessitated further optimization which was achieved by using a gradient block (see section 2.8.6.2.) to pinpoint the optimal temperature for enhanced amplification of the target fragment for rat gut. For brain tissue the optimal amplification temperature was 57^{o}C (Figure 3.8. A), whereas rat gut tissue yielded stronger PCR signals at lower temperatures (Figure 3.8. B). Final optimising to reduce the amplification of non-specific products was done, with the best results for rat gut being obtained at 52-54 ^{o}C (Figure 3.8.C).

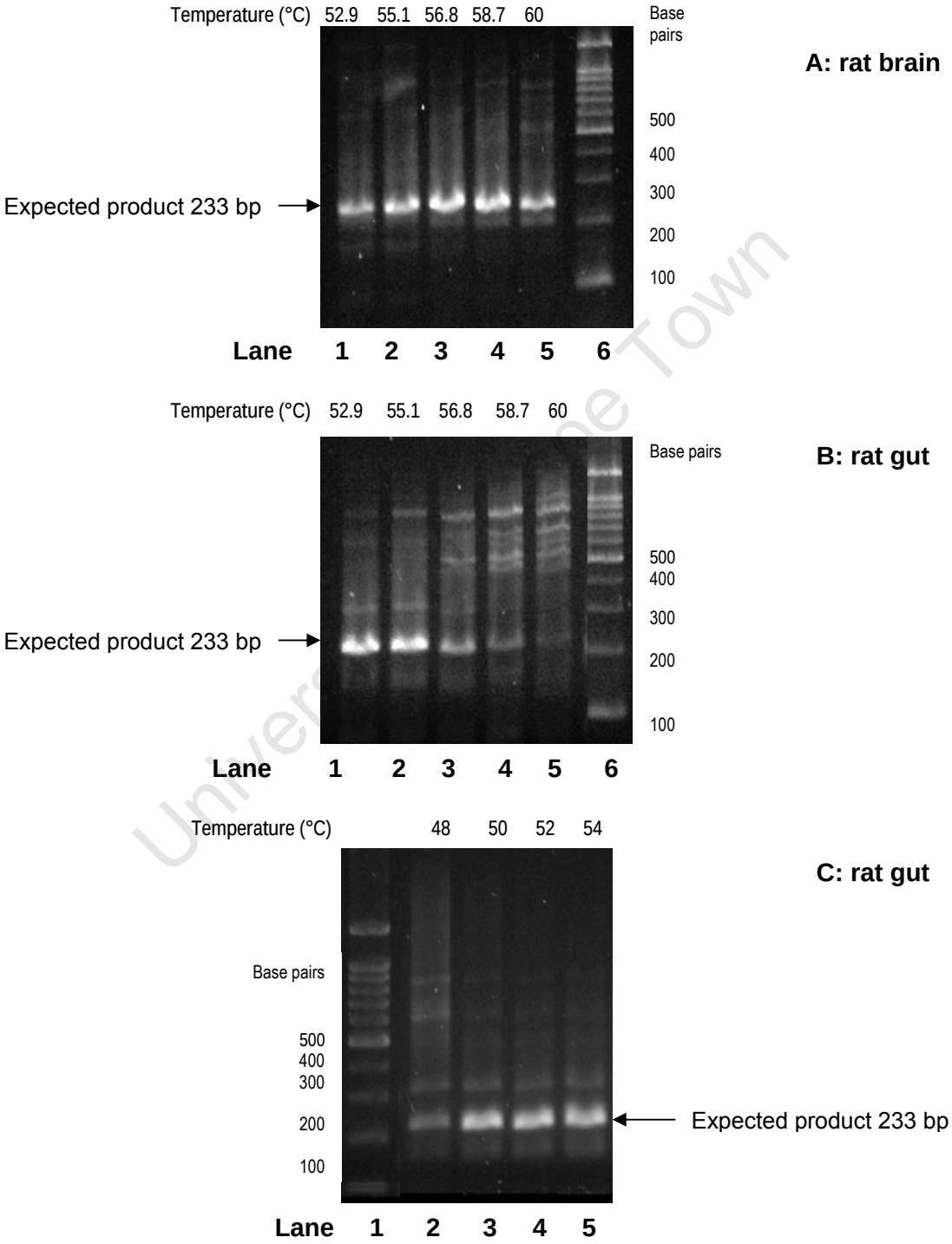


Figure 3.8. Gradient PCR analysis of rat brain (A) and gut (B and C) cDNA

For both A and B: Lane 6: molecular weight marker. For C: Lane 1: molecular weight marker

As stated previously, the primer design could not distinguish between contaminating gDNA in specimen and cDNA derived from the RNA of interest. Thus, to finally confirm that the 233 bp product was derived from mRNA, reactions were performed without the RT step. The absence of specific product in the RT-negative lanes (Figure 3.9. lanes 1 and 2) confirmed that the PCR product was derived from the mRNA.

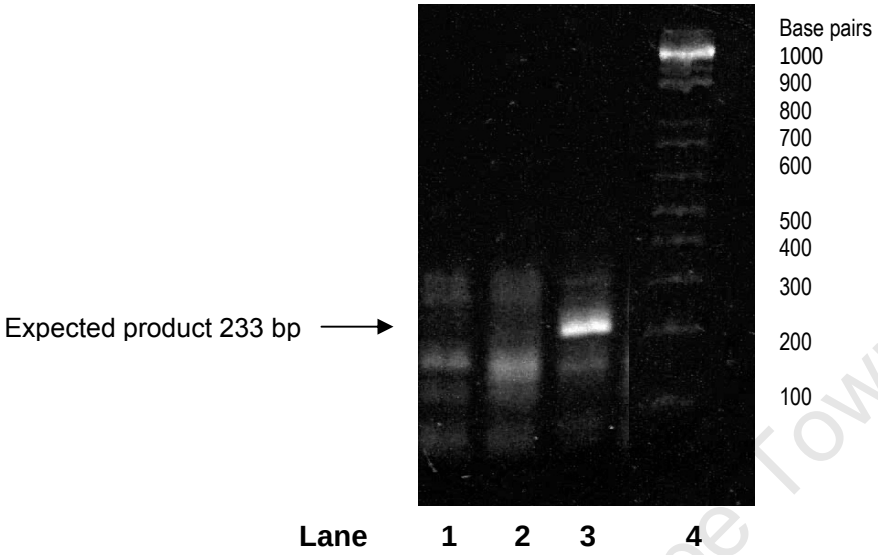


Figure 3.9. PCR products of cDNA synthesis reactions and control reactions for CRF

Lane 1: water blank (no RNA), Lane 2: Rat gut RNA (no RT), Lane 3: Rat gut RNA (RT), Lane 4: molecular weight marker

However, in all lanes, other products were seen. To further minimise these, additional optimisation was carried out. Results showed that optimal conditions included a FailSafe™ buffer concentration of 1X and a temperature of 57°C (Figure 3.10.C lane 5). A non-specific product of about 300 bp could not be eliminated.

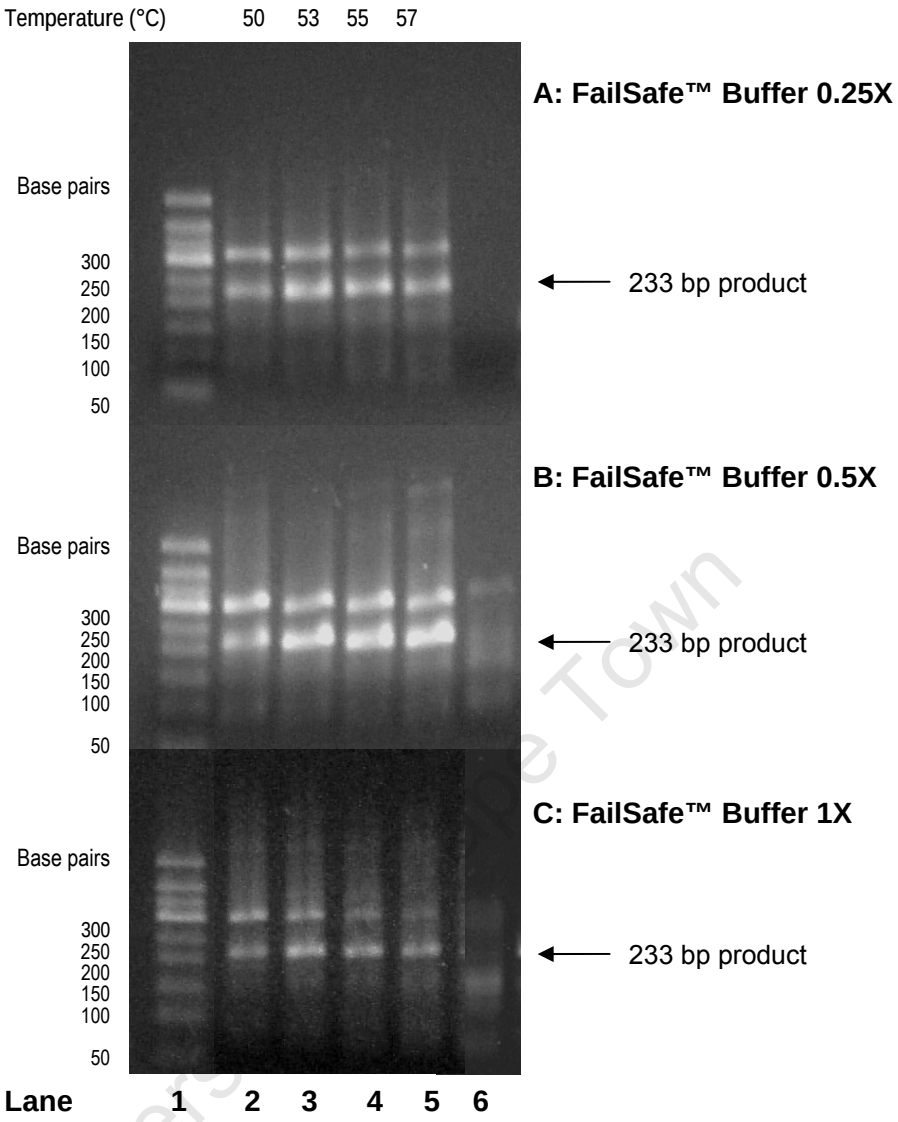


Figure 3.10. Gradient PCR analysis of rat gut cDNA using different buffer concentrations

For A, B and C Lane 1: 50 bp molecular weight marker.

For A, B and C Lanes 2-5: rat gut cDNA amplified at different PCR annealing temperatures

For A, B and C Lane 6: rat gut (no RT)

Restriction enzyme digest

To confirm that the 233bp PCR product was indeed CRF, DNA sequencing and restriction enzyme digestion was carried out on purified product. The rat CRF sequence amplified by the set of primers used in this study is known to have a PstI restriction site at positions 18 and 27 (http://rna.lundberg.gu.se/cutter2/). The results of that restriction digest are shown below in Figure 3.11. A and B, and confirm that the PCR product is in fact the desired CRF amplicon.

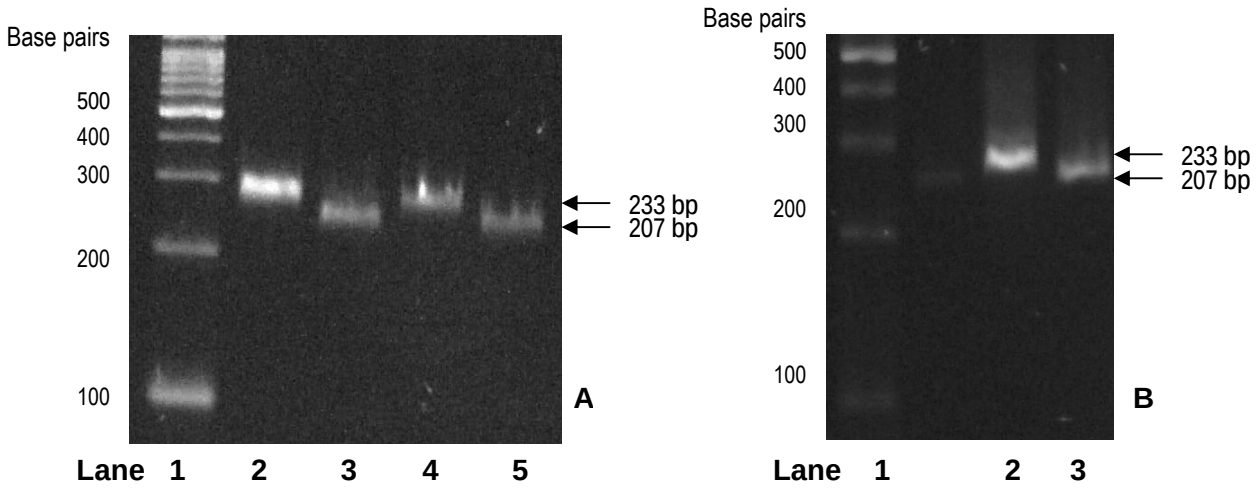


Figure 3.11. Restriction enzyme digest of 233 bp PCR product from rat tissues

For A:

Lane 1: Molecular weight marker, Lane 2: rat brain undigested product, Lane 3: rat brain showing product after PstI digestion, Lane 4: rat hypothalamus undigested product, Lane 5: rat hypothalamus showing product after PstI digestion

For B:

Lane 1: Molecular weight marker, Lane 2: rat gut undigested product, Lane 3: rat gut showing product after PstI digestion

cDNA sequencing

As a final verification that the band seen on gel electrophoresis was the desired band, cycle sequencing was performed on the PCR product, which was excised from the gel and cleaned as described in section 2.8.6.4. Cycle sequencing was performed in one direction using the sense primer used in PCR. The products were then ethanol precipitated before they were electrophoresed and analyzed on the ABI Prism 3100 system (Figure 3.12.). The analysis program provides an electropherogram showing peaks of different colours for the four bases which are translated into a base sequence that can be identified in an online program such as NCBI BLAST. The BLAST search confirmed that the PCR product amplified in all three rat tissues was indeed rat CRF (Table 3.4).

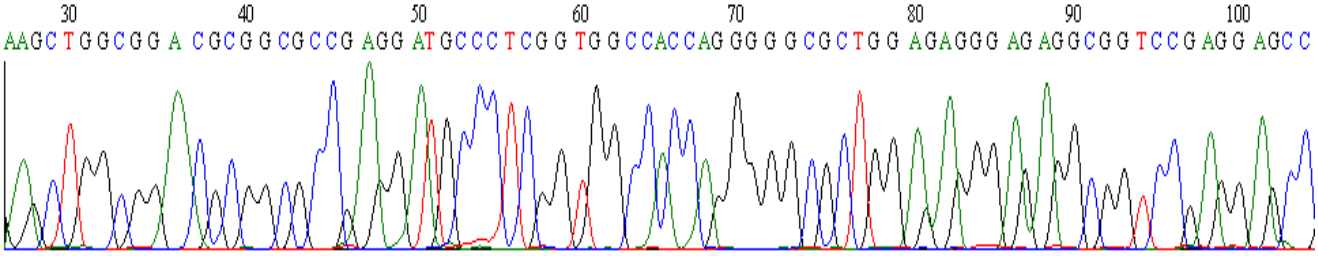


Figure 3.12. Electropherogram of base sequence of purified 233 base pair PCR product

Table 3.4. Sequence alignment of purified PCR product with NCBI accession number NM_031019 identified as *Rattus norvegicus* CRF mRNA

Base		Base
	AGCTGGCGG-ACGCGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAGAGGGAGAGGCGGTCCGAGGAGCCG	
540	AGCTGGCGGAACGCGGCGCCGAGGATGCCCTCGGTGGCCACCAGGGGGCGCTGGAGAGGGAGAGGCGGTCCGAGGAGCCG	619
Key:		
Base sequence of purified 233 bp PCR product		
Base sequence of rat CRF mRNA (NCBI Accession number NM_031019)		

3.3.2. Expression of CRF mRNA in human tissue

All intestinal tissue specimens for this study had been frozen and stored at -80°C as described. RNA extracted from a first batch of these specimens and analysed by gel electrophoresis suggested that the quality of RNA was not very good. For example, see Figure 3.13. lanes 1 and 2. Because the study design did not allow one to obtain new specimens from patients, it was vital that the RNA extraction and all subsequent steps be optimised to the maximum. Therefore, a series of technique optimising experiments were conducted on tissue from an elective patient that had been flash-frozen but not stored. As can be seen in Figure 3.13. (lanes 3, 4 and 5), the quality of RNA extracted from this tissue appeared much better, with clear ribosomal bands.

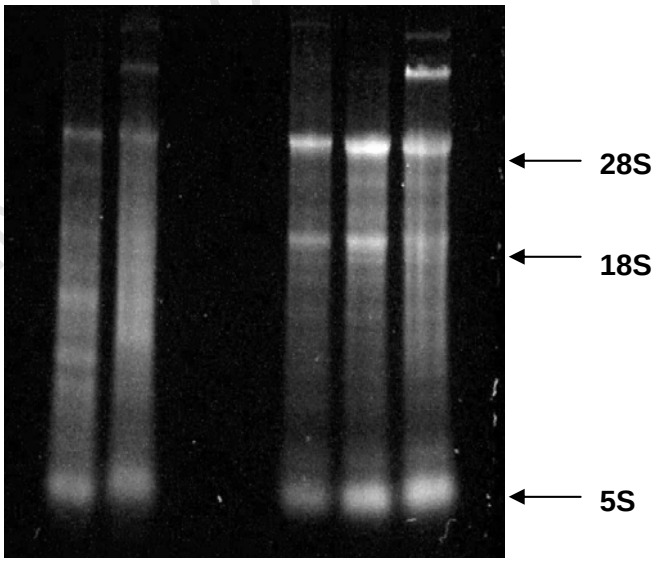


Figure 3.13. RNA quality from human gut tissue stored and homogenised by different methods

Lane 1 and 2: RNA from human gut tissue stored at -80°C for several months

Lane 3: RNA from human gut tissue extracted immediately and homogenised using Polytron

Lane 4: RNA from human gut tissue extracted immediately and homogenised using pestle and mortar followed by Polytron

Lane 5: RNA from human gut tissue extracted immediately and homogenised using pestle and mortar

Using this RNA, gradient RT-PCR was carried out. As seen in Figure 3.14., PCR products obtained from amplification of human samples appeared smaller than the known CRF product in rat gut, with band sizes of between 100 and 200bp (lanes 6-9). It has been previously reported that CRF expressed in different tissue types may be of differing sizes (Thompson et al 1987). To establish whether these smaller fragments were CRF or not, they were excised, cleaned and DNA sequenced. NCBI BLAST search revealed, however, that the product was not CRF.

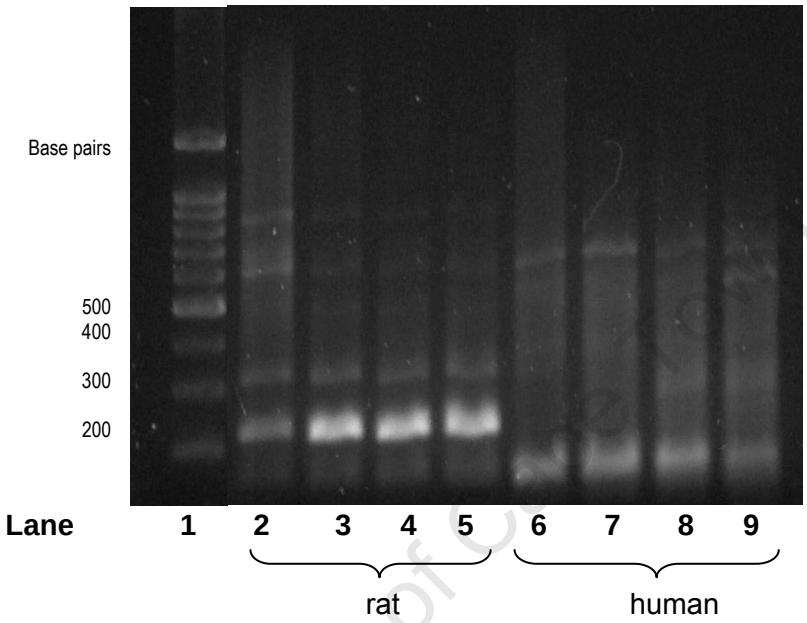


Figure 3.14. Gradient PCR analysis of cDNA for rat gut and human gut

Lane 1: molecular weight marker, Lane 2: 48°C, Lane 3: 50°C, Lane 4: 52°C, Lane 5: 54°C (all lanes rat gut)
Lane 6: 48°C, Lane 7: 50°C, Lane 8: 52°C, Lane 9: 54°C (all lanes human gut)

Further gradient RT-PCR analysis was performed using a very wide temperature range of 46-63°C. The result of this analysis is shown in Figure 3.15. below.

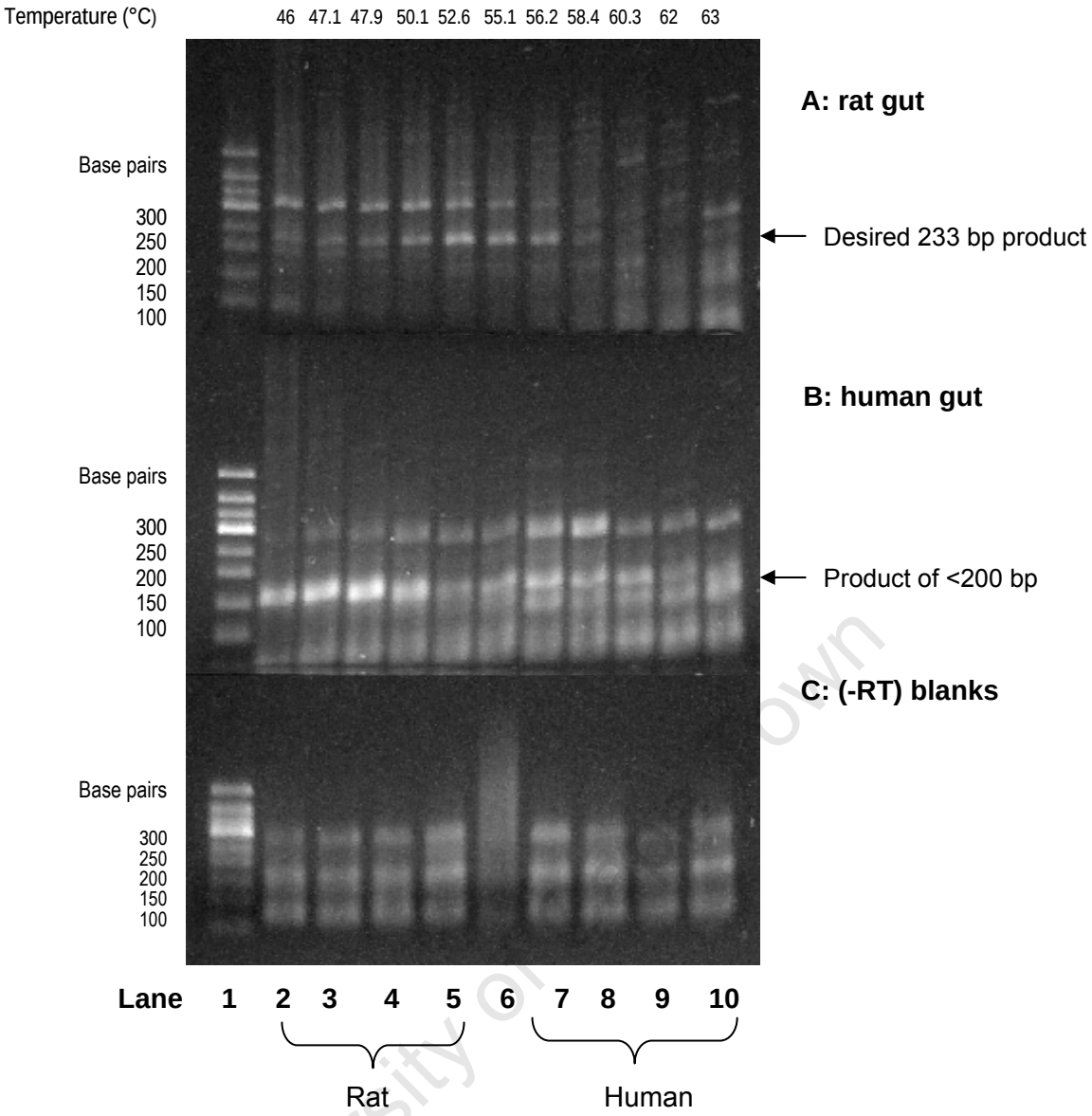


Figure 3.15. Gradient PCR analysis of rat and human gut cDNA

For A, B and C Lane 1: molecular weight marker.

For A: Lanes 2-10: rat gut cDNA amplified at different PCR annealing temperatures

For B: Lanes 2-10: human gut cDNA amplified at different PCR annealing temperatures

For C: Lanes 2-5: rat gut (no RT) Lanes 6-10: human gut (no RT)

These results indicate the following: Firstly, the wide gradient confirms that the RT-PCR conditions previously used for rat gut were optimal, with the brightest product band of the 233 bp size appearing in the 52-55°C range. Secondly, at none of the temperatures was a 233 bp product amplified for human gut. Across the entire temperature range (Fig 3.15. lanes 2-10) a product of between 150 bp and 200bp consistently resolved and was also present in the –RT blanks. As indicated above, DNA sequencing indicated that this product was not CRF. In the final experiments, different concentrations of FailSafe™ buffer at different temperatures were tested, but, yet again, CRF was not amplified and the smaller product consistently seen was again visible. See Figure 3.16. (A and B lanes 3-6).

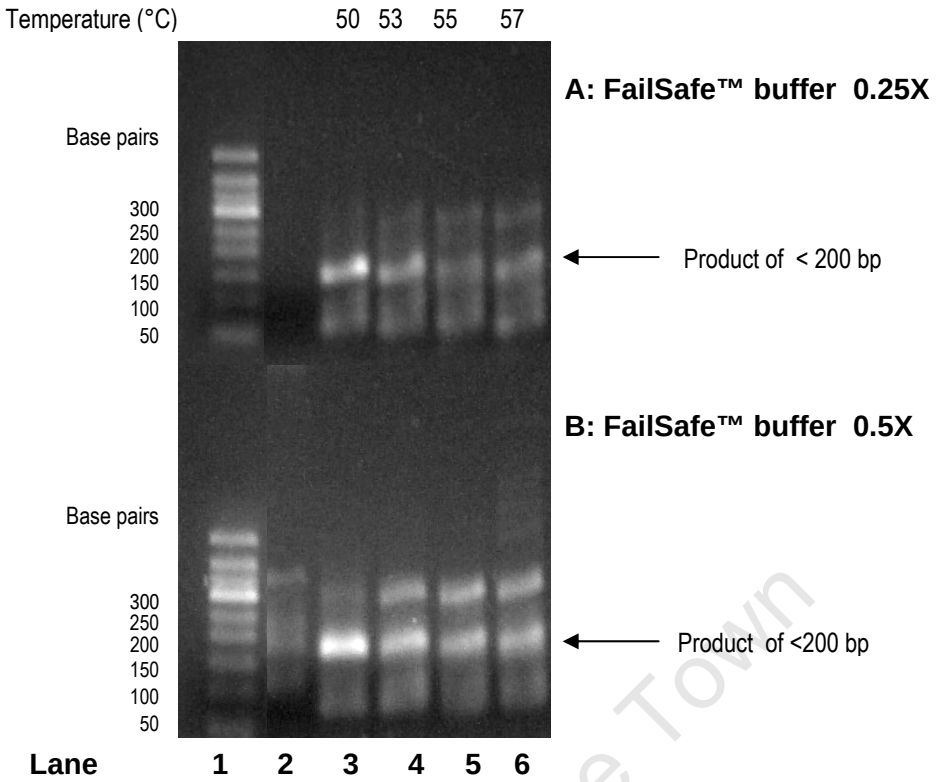


Figure 3.16. Gradient PCR analysis human gut cDNA using different buffer concentrations

For A and B Lane 1: molecular weight marker; lane 2: human gut cDNA (no RT); Lanes 3-6: human gut cDNA at different PCR annealing temperatures

In summary, CRF mRNA could not be detected in either the good RNA sample, or in any sample tested. This suggested that either CRF is not expressed in human intestinal tissue, or the technique is inadequate. As a final attempt to provide insight into this problem, the quality of the RNA was tested by RT-PCR analysis of two housekeeping genes – GAPDH (high levels of expression in most tissues) and GUS1B (low levels of expression).

Amplification of human GAPDH resulted in RT-PCR products of the expected sizes (about 100 bp, see arrow). See Figure 3.17. lanes 2 and 3 below. Interestingly, the stored samples from the trauma patients also showed bands for GAPDH (lanes 8 and 9). A very small product (<50 bp) resolved in all lanes, except that of the molecular weight marker. This was presumably a primer dimer.

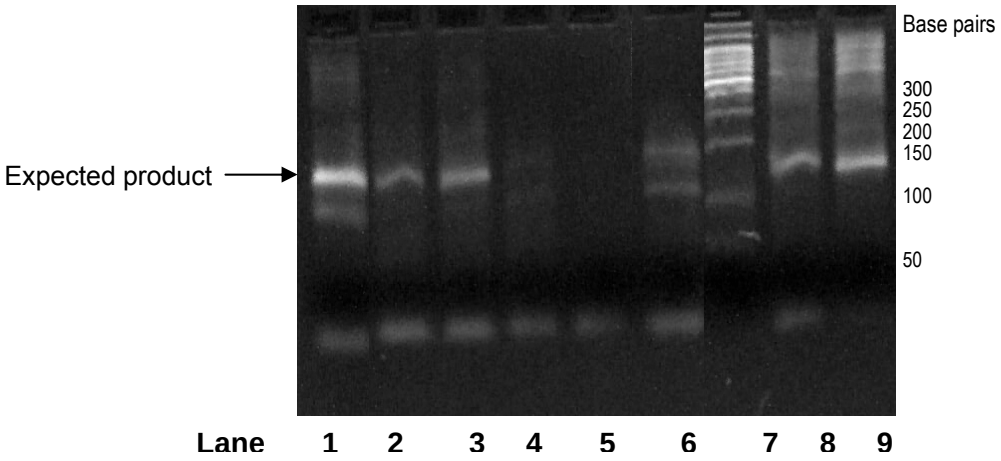


Figure 3.17. PCR analysis of GAPDH in human gut cDNA

Lane 1: control cDNA sample; Lane 2: DNAse-treated (30 minutes) cDNA from human gut RNA from elective patient; Lane 3: DNAse-treated (15 minutes) cDNA from human gut RNA from elective patient; Lane 4: human gut from elective patient (no RT); Lane 5: water blank; lane 6: human gut from elective patient (no RNA); Lane 7: molecular weight marker; Lane 8: stored human gut from trauma patient; Lane 9: stored human gut from trauma patient

Similarly, GUS1B (about 100 bp, see arrow) was expressed in all samples, except for one of the stored specimens (Figure 3.18.)

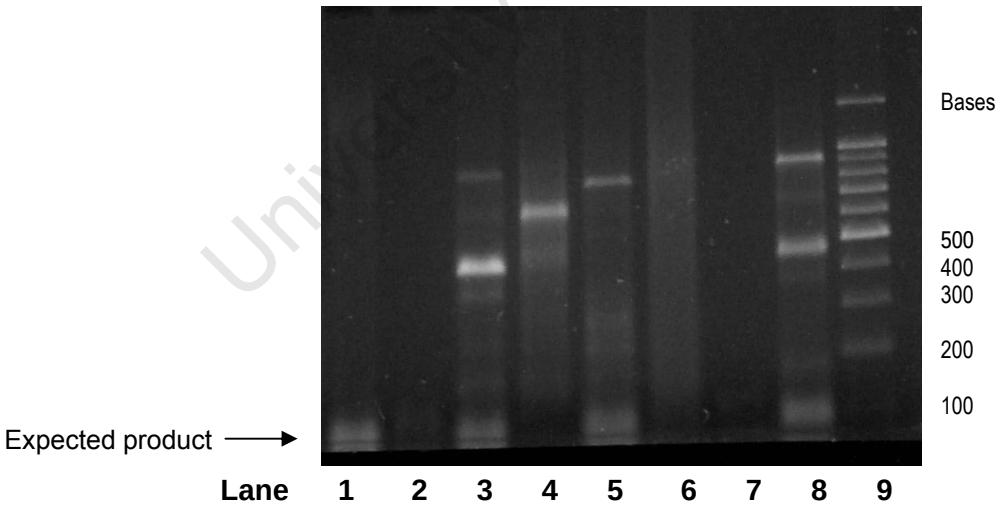


Figure 3.18. PCR analysis of GUS1B for human gut cDNA

Lane 1: elective patient sample treated with DNAse; Lane 2: elective patient sample treated with DNAse (no RT); Lane 3: cDNA control; Lane 4: stored human gut from trauma patient; Lane 5: stored human gut from trauma patient; Lane 6: water blank; Lane 7: elective patient sample treated with DNAse (no RNA); Lane 8: cDNA control; Lane 9: molecular weight marker

The detection of GUS1B expression would seem to suggest that the quality of RNA is adequate for the analysis of low prevalence mRNAs. One is therefore led to conclude that perhaps CRF is simply not expressed in human intestinal tissue.

3.4. Gastrointestinal physiology and symptoms

3.4.1. Intestinal permeability

One of the study objectives was to describe gut function and to investigate any relationship between CRF and gut physiological function. The first indicator of gut function investigated was intestinal permeability. This was carried out on the first post-operative day by means of a combined monosaccharide (mannitol)/disaccharide (lactulose) sugar absorption test. Permeability of the small intestine could be tested in 7 trauma and 13 elective patients. In the rest of the patients, various clinical circumstances prevented testing and in 1 patient the urine specimen was discarded in error. With lactulose:mannitol ratios of 10-20 times normal both trauma and elective patients displayed similarly enhanced intestinal permeability to sugar probes. See Table 3.5.

Table 3.5. Median (IQR) indicators of intestinal permeability

	Trauma (n=7)	Elective# (n=13)	p-value
Urinary lactulose (% loading dose)	1.08 (0.006-1.27)	0.96 (0.28-1.5)	0.97
Urinary mannitol (% loading dose)	3.7 (1.9-4.3)	2.3 (1.3-5.9)	0.5
Urinary lactulose: mannitol*	0.428 (0.09-0.67)	0.64 (0.39-1.1)	0.4

* upper limit of normal = 0.03 based on data from several hundred healthy persons (Faries et al, 1998)

* highly increased intestinal permeability = 0.03-0.1

sub-group analysis of elective patients revealed significantly higher lactulose:mannitol in jaundiced vs non-jaundiced patients (p=0.016). However, neither elective sub-group had a lactulose:mannitol that was significantly different from trauma patients.

Both indicators of transcellular (% urinary excretion of mannitol) and paracellular (% urinary excretion of lactulose) intestinal absorption were increased in both patient groups, with no inter-group difference. The hyper-permeability-defining variable urinary lactulose: mannitol was also similar in both groups. Only 1 patient in each group had a result that fell within the limits of the reference range based on data from healthy volunteers (inter-group frequency difference p= NS, Fisher's exact). Five of the 7 trauma patients (71%) and 11 of the 13 (85%) elective patients (p= NS, Fisher's exact) had urinary lactulose:mannitol values in the range of 0.03-0.1 considered 'highly increased permeability' (Faries et al, 1998).

The lactulose:mannitol results for both groups were higher than published reports of tests involving healthy volunteers. In order to identify whether this increase was statistically significant, the difference between published mean values and mean values for the study groups was computed (see Table 3.6.). The results indicate that both elective and trauma patients have significantly increased intestinal

permeability compared to healthy volunteers. The inter-group mean values between trauma and elective patients were not significantly different.

Table 3.6. Mean ± sd of urinary lactulose: mannitol of study groups and healthy volunteers

	Trauma (n=7)	Elective[#] (n=13)	Healthy volunteers	p-value
Urinary lactulose: mannitol	0.56 ± 0.62	0.76 ± 0.52	0.035 ± 0.005 (n=11, Ziegler, 1988)	0.0001 vs elective 0.011 vs trauma
			0.017 ± 0.002 (n=15, Deitch, 1990)	<0.00001 vs elective 0.002 vs trauma
			0.017 ± 0.003 (n=10, LeVoyer, 1992)	0.0002 vs elective 0.013 vs trauma

sub-group analysis of elective patients: both jaundiced and non-jaundiced elective patients had lactulose:mannitol ratios statistically higher than healthy volunteers.

No association was found between plasma or tissue CRF concentrations and intestinal permeability in either study group. However, in the trauma group duration of surgery was positively and significantly correlated with urinary lactulose: mannitol (Spearmans' R = 0.76, p=0.045). This could be the result of more handling and manipulation of the intestines during the operative procedure. A strong negative association was also found between minimum recorded systolic blood pressure and both lactulose: mannitol ratio and percentage urinary excretion of lactulose dose. These are both indicators of enhanced permeability, and this result demonstrates the link between shock and loss of intestinal mucosal barrier integrity. This is graphically shown below in Figure 3.19. Additionally, in the trauma group there was a strong positive and significant correlation between volume of intravenous fluids received in the first 24 hours and urinary lactulose (Spearman's R = 0.89, p=0.006). This was not found in elective patients.

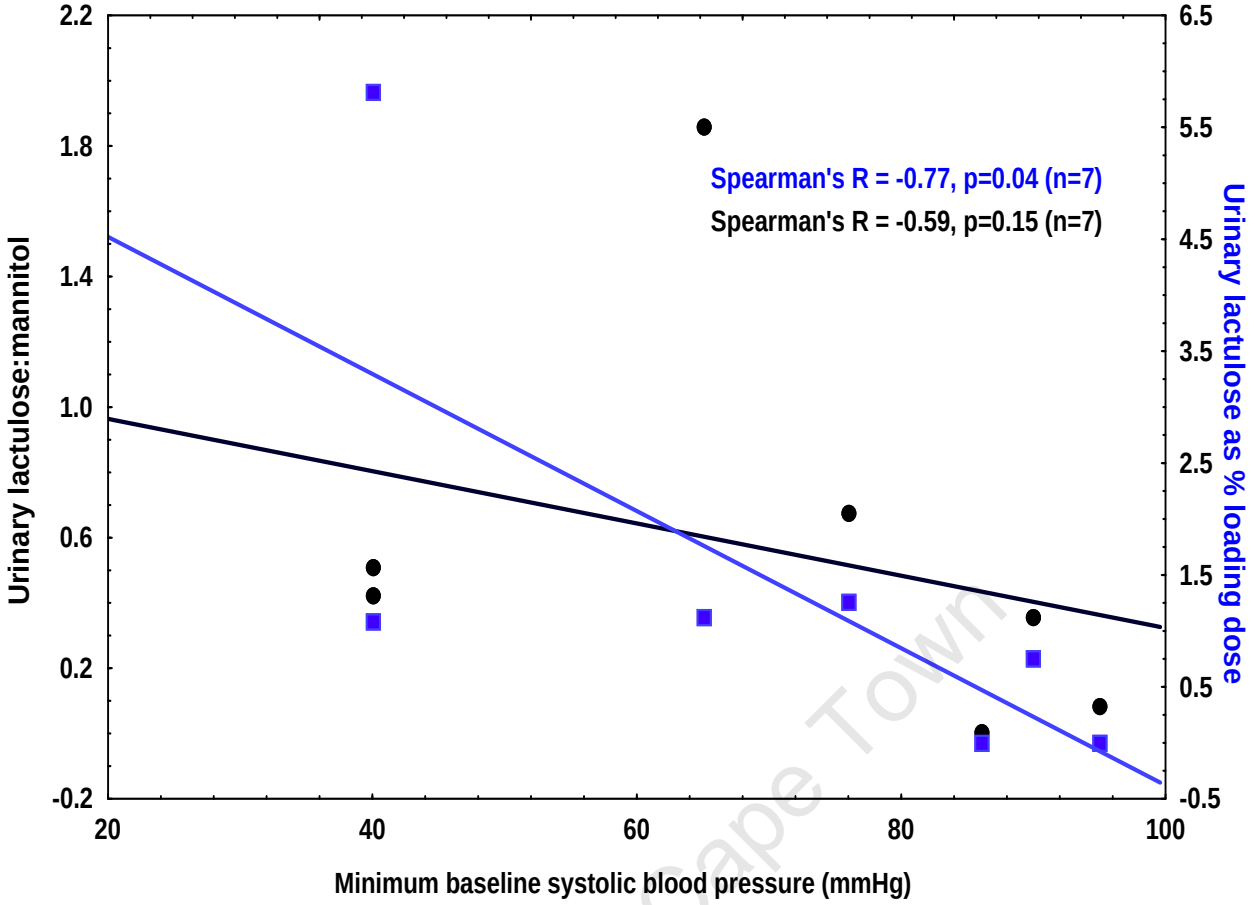


Figure 3.19. Minimum systolic blood pressure (mmHg) vs intestinal permeability indicators

To summarise, the enhanced intestinal permeability was unrelated to tissue CRF levels, but was associated with shock and exposure to surgery in trauma patients.

3.4.2. Gastric emptying

The rate of gastric emptying was evaluated on the first post-operative day in both patient groups. The test procedure began approximately 24 hours following the surgical procedure, and was performed concurrently with the intestinal permeability testing. Gastric emptying rate was tested by means of a paracetamol absorption test. The test was not performed where a patient had a gastro-enterostomy as part of the elective surgical procedure.

Figure 3.20. indicates the plot of paracetamol concentration versus time for each of the groups. Trauma patients are shown in black dots and elective patients in blue triangles. The graph indicates the slow paracetamol absorption seen in both groups.

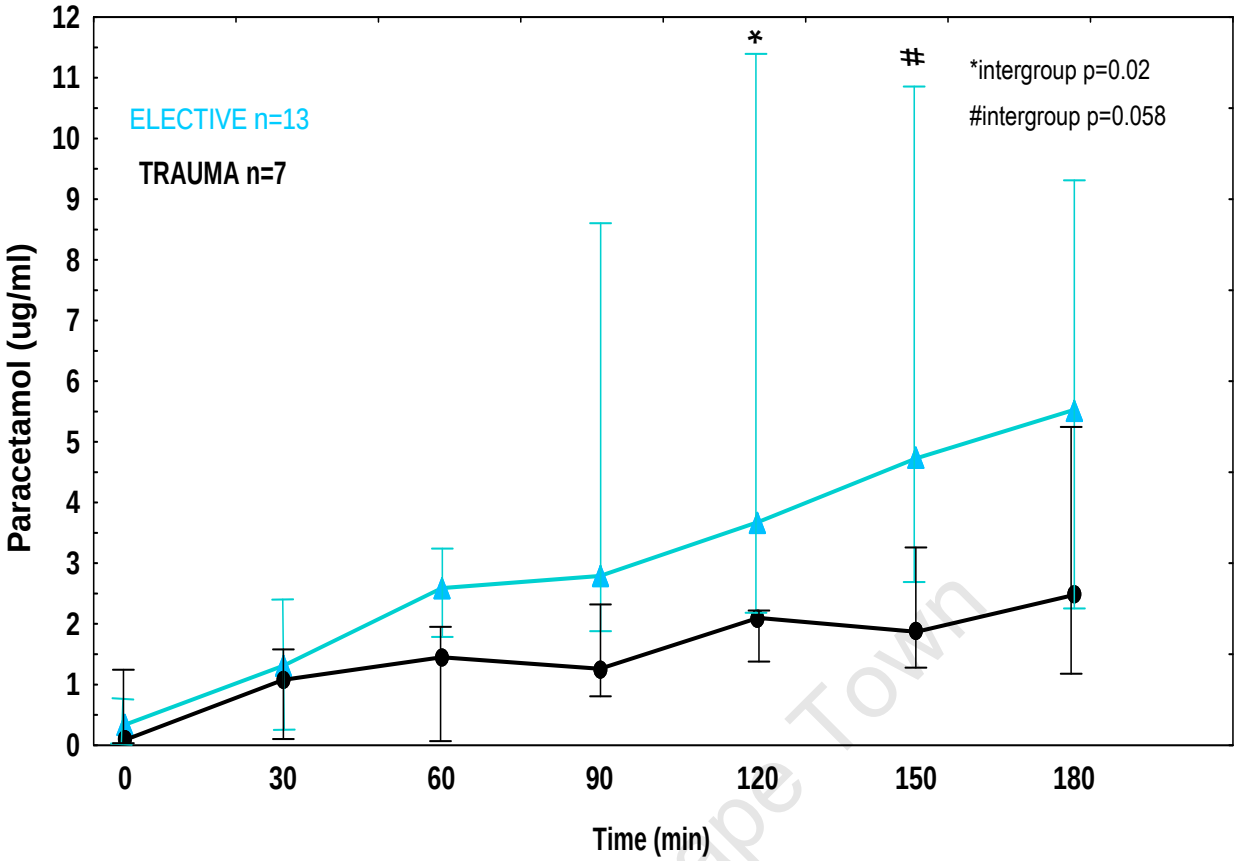


Figure 3.20. Median (IQR) paracetamol concentrations in elective and trauma groups

The trauma group in particular showed a very minimal rise from baseline in paracetamol concentration throughout the test period. In fact, by repeated measures ANOVA the overall difference in the rate of rise in paracetamol concentration was not statistically different between the 2 groups (p=0.16). However, the interaction effect approached significance (p=0.057). Post-hoc analysis revealed that for the elective group the paracetamol concentration at all time points from 90 minutes onward were significantly higher than baseline (p<0.0006 in all cases). This was not seen in the trauma group, and paracetamol levels remained statistically the same as baseline concentrations (p=NS) throughout the entire test, indicating significantly delayed gastric emptying. A significant inter-group difference occurred at 120min and approached significance at 150min (p=0.02 and p=0.058).

As shown in Table 3.7. both groups had a similarly low C_{max}, late T_{max}, and low AUC at the various time points. All of these indicate poor gastric emptying in both study groups. However, there was a trend among the trauma group for slower gastric emptying after 90 minutes and at 120 minutes the AUC for the trauma group was significantly lower compared with the elective patients.

Table 3.7. Median (IQR) parameters of gastric emptying

	Trauma (n=6)	Elective (n=13)	p-value
Cmax (ug/ml)	2.86 (2.1-5.3)	4.59 (2.6-12)	0.2
Tmax (min)	165 (120-180)	150 (120-180)	0.7
AUC_{60} (ug/ml/min)	72 (3-90)	72 (48-130)	0.36
AUC_{90} (ug/ml/min)	122 (18-136)	159 (92 -279)	**0.12**
AUC_{120} (ug/ml/min)	165 (64-203)	240 (150-630)	**0.02**
AUC_{180} (ug/ml/min)	275 (198-374)	466 (192-1318)	0.4

Cmax – maximum concentration
Tmax – time to maximum concentration
AUC_{60} – area under the concentration vs time curve after 60min
AUC_{90} – area under the concentration vs time curve after 90 min
AUC_{120} – area under the concentration vs time curve after 120min
AUC_{180} – area under the concentration vs time curve after 180min

The AUC and C_{max} values in both patient groups appeared lower, and the T_{max} longer than previous published reports for both stable patients and healthy volunteers. Additionally, our AUC_{60} values appeared much lower than the 600 mg/min/l defined as “normal” by Avrahami et al (1999). In order to compare our results with those published in literature mean ± sd values were calculated for the study groups and statistically compared with published data. Those results are shown in Table 3.8. below.

Table 3.8. Mean ± sd of gastric emptying parameters of study groups and published data

	Trauma (n=6)	**Elective (n=13)**	Published values	p-value
C_{max} (ug/ml)	4 ± 3.8	7.6 ± 6.0	15.6 ± 0.85 Healthy volunteers n=18 (Takeda et al, 1999)	<0.00001 vs elective <0.00001 vs trauma
			23.9 ±8.7 General hospital patients, n=14 (Heading et al, 1973)	<0.00001 vs elective <0.00001 vs trauma
			5.1 ± 2.8 Post-operative cardiac patients n=13 (Goldhill et al, 1995)	0.186 vs elective 0.48 vs trauma
			17 ± 11 Post-thoractomy patients on epidural analgesia n=11 (Guha et al, 2002)	0.014 vs elective 0.014 vs trauma
T_{max} (min)	150 ± 37.9	143 ± 39	68.3 ± 4.3 Healthy volunteers n=18 (Takeda et al, 1999)	<0.00001 vs elective <0.00001 vs trauma
			83.6 ± 45.8 General hospital patients, n=14 (Heading et al, 1973)	<0.0011 vs elective <0.0049 vs trauma
			225.4 ± 152.5 Post-operative cardiac patients n=13 (Goldhill et al, 1995)	0.07 vs elective 0.255 vs trauma
AUC_{60} (ug/ml/min)	97.4 ± 145.4	132.4 ± 185.1	484.2 ± 31.7 Healthy volunteers n=18 (Takeda et al, 1999)	<0.00001 vs elective <0.00001 vs trauma
			131 ± 90.2 Post-operative cardiac patients n=13 (Goldhill et al, 1995)	0.98 vs elective 0.54 vs trauma
AUC_{90} (ug/ml/min)	169.4 ± 245.9	261.5 ± 310.9	920.3 ± 51.9 Healthy volunteers n=18 (Takeda et al, 1999)	<0.00001 vs elective <0.00001 vs trauma
AUC_{120} (ug/ml/min)	249.3 ± 340.8	451.7 ± 466.6	1408 ± 880 Post-thoracotomy patients on epidural analgesia n=11 (Guha et al, 2002)	0.0026 vs elective 0.0079 vs trauma

Compared with both healthy volunteers, both the trauma and elective patient groups in this study had significantly impaired gastric emptying. When compared with post-operative data from other patient

groups the patient groups in this study were not different from cardiac patients in terms of C_{max} or $T_{max.}$ However, both trauma and elective patients had significantly lower C_{max} and higher $T_{max.}$ when compared with convalescing general ward in-patients, and lower C_{max} compared with post-thoracotomy patients who were managed with post-operative epidural care. Neither trauma nor elective patients in this study had an AUC_{60} that was significantly different from that of post-operative cardiac patients. Compared with post-thoracotomy patients receiving epidural analgesia, however, both groups had a significantly retarded $AUC_{120.}$ In all cases the mean values of the 2 groups included in this study were not statistically different in terms of any of these parameters.

Among the trauma group the AUC_{180} showed a trend toward being negatively correlated with baseline plasma CRF concentration (Spearman's R = -0.66, p=0.15). Baseline plasma CRF was also strongly positively correlated with paracetamol T_{max}, with a trend to statistical significance (Spearman's R = 0.7, p=0.12). These results suggest that higher circulating CRF concentrations are related to more impaired gastric emptying. AUC_{180} was associated with a longer ICU stay (Spearman's R = 0.88, p=0.019), possibly indicating worse outcomes for those with delayed gastric emptying.

In the elective group 24 hour post-operative plasma CRF concentration was negatively correlated with paracetamol C_{max} (Spearman's R = -0.51, p=0.07) and AUC_{180} (Spearman's R = -0.48, p=0.09), and significantly so with AUC_{120} (Spearman's R = -0.5, p=0.04). These results infer that higher circulating levels of CRF are associated with worse gastric emptying, as hypothesised.

The strongest indicators of poor gastric emptying, however, were minimum systolic blood pressure during the first 24 hours and morphine use. In the two groups combined minimum systolic blood pressure correlated strongly negatively and significantly with T_{max} (Spearman's R = -0.73, p=0.0003), meaning that the lower the systolic blood pressure the more delayed the gastric emptying time.

The total morphine received via all routes of administration in the first 24 hours post-operatively (day 0) was a median (IQR) amount of 10 (6-30.5) mg in elective patients (including morphine in epidural) and 40 (24.5-53) mg in trauma patients (combined intravenous and intramuscular). This is a significant group difference (p=0.01). While the total dosage of morphine on day 0 was not correlated with gastric emptying parameters in either of the 2 study groups separately, a significant negative correlation was seen for the combined groups (n=18) between total day 0 morphine dosage and Cmax (Spearman's R = -0.51, p=0.03), when an outlier was excluded. Similarly, the negative correlation between total day 0 morphine dosage and AUC_{180} showed a trend toward significance in the combined groups as well (Spearman's R = -0.44, p=0.06). These correlations as can be seen in Figures 3.21. and 3.22. below.

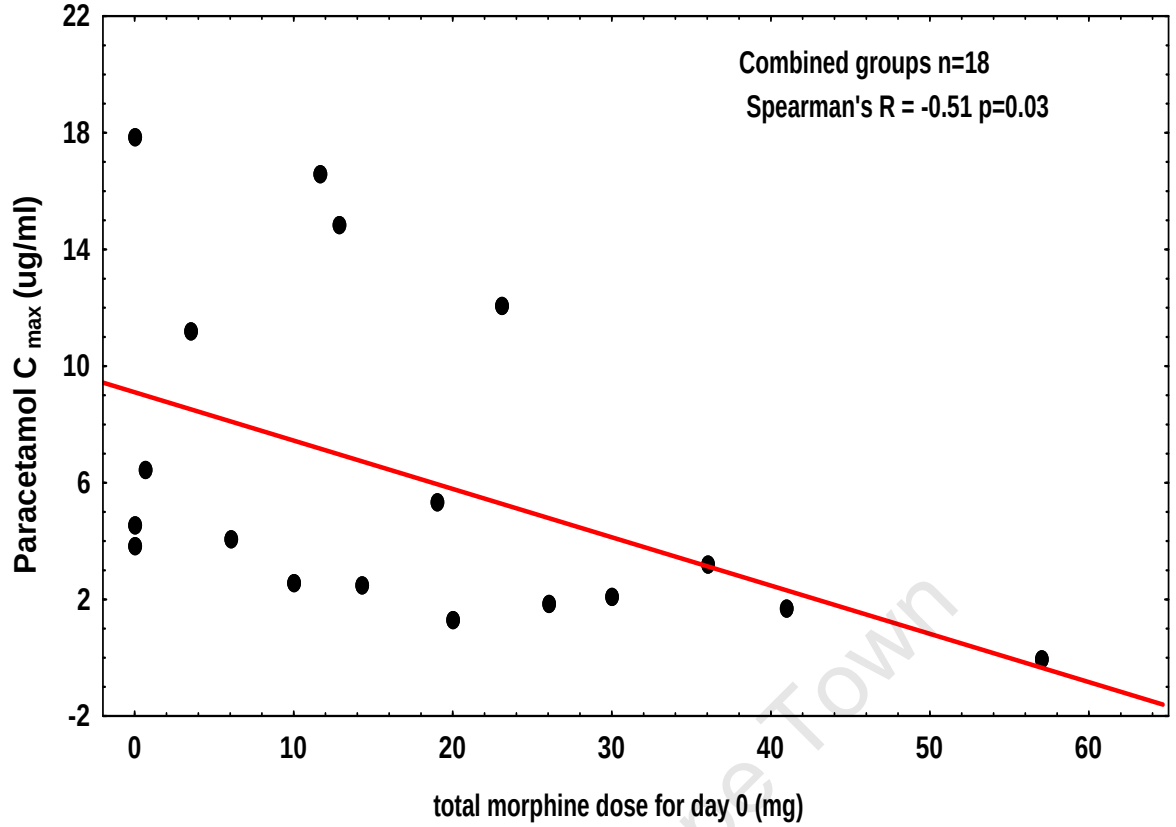


Figure 3.21. Total day 0 morphine dose (mg) vs paracetamol C_{max} (ug/ml)

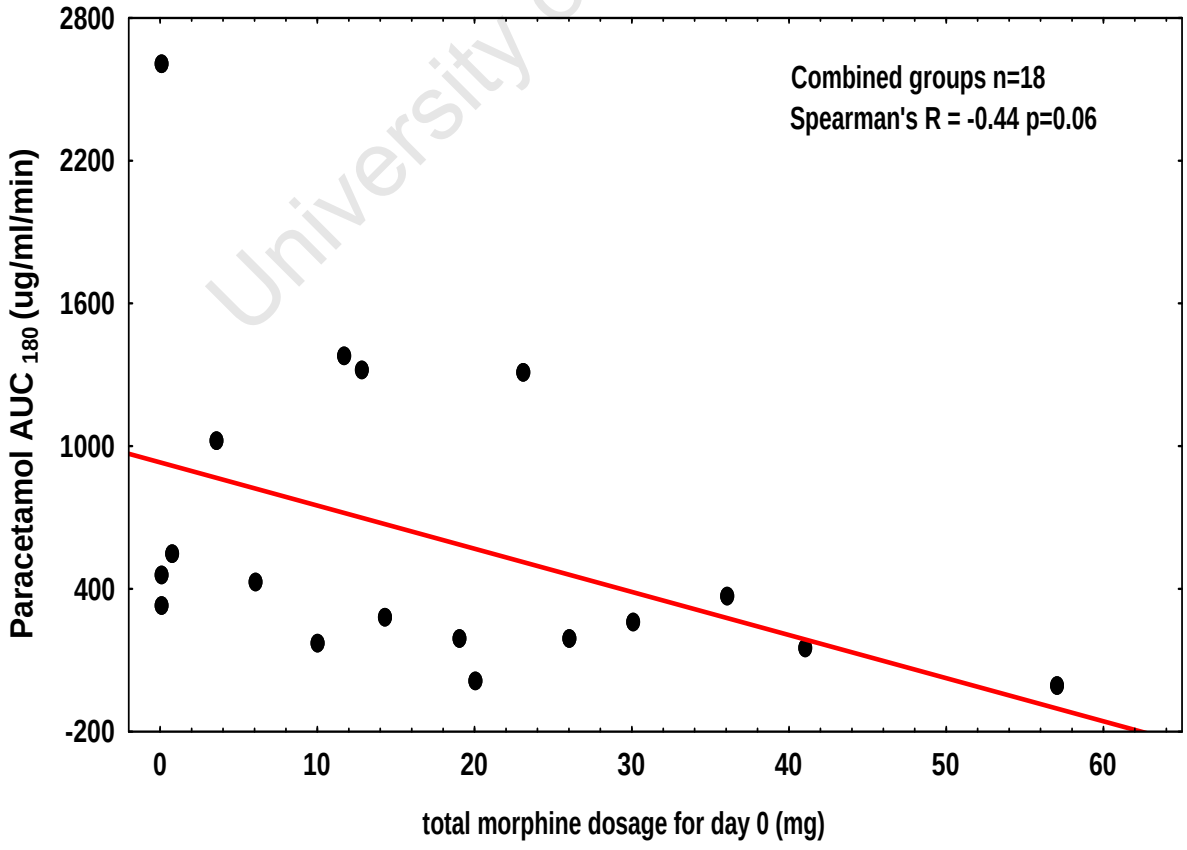


Figure 3.22. Total day 0 morphine dose (mg) vs paracetamol AUC_{180} (ug/ml/min)

The same significant negative relationship was found when total morphine dose was correlated with AUC_{90} (Spearman's R = -0.49, p=0.03) and AUC_{120} (Spearman's R = -0.51, p=0.027) using combined data from both study groups (n=18). The epidural route of analgesia was not associated with markers of gastric emptying. However, the combined dose of morphine administered by non-epidural (intravenous and intramuscular) routes was also negatively and significantly correlated with both C_{max} (Spearman's R = -0.53, p=0.02) and AUC_{180} (Spearman's R = -0.46, p=0.05) in the 2 groups combined (n=18).

3.4.3 Gastrointestinal symptoms

Diarrhoea in the first 3 post-operative days was observed in 1 elective patient and 0 trauma patients (Fisher's exact, p=NS). Median episodes of vomiting within the first 3 post-operative days was 0 (0-2) in elective patients and 0 (0-1) in the trauma group (Mann-Whitney p= 0.6). The median number of times within the first 3 post-operative days when the 24 hour nasogastric drainage volume exceeded 250ml was 1.5 (0.5-2) in electives and 1 (0-1.5) in trauma patients (Mann-Whitney p=0.4). The median volume of nasogastric drainage in the first 24 hours was 50 (0-200) ml in the elective group and a statistically similar 122.5 (50-300) ml in trauma patients (Mann-Whitney p= 0.36). However, in neither group was the dosage of morphine associated with number of vomiting episodes or nasogastric drainage volumes. There were no associations between plasma or tissue CRF and any gastrointestinal symptoms.

3.5. Histology

3.5.1. Mast cell quantification

Based on the hypothesis that stress and CRF release are associated with mast cell infiltration, an objective was to quantify mast cell numbers in resected gut tissue obtained from trauma and elective surgery patients at the time of surgery. This was done using light microscopy and counting mast cell numbers in all fields using a calibrated graticule. Full thickness tissue sections were viewed and counted by histological layer i.e. mucosa, submucosa, muscularis and serosa.

Mast cells were detected in at least one gut tissue layer in all trauma subjects. However, in 5 of the 17 elective patients no mast cells were detected on histological examination. There was a statistical trend toward significance for this difference in the frequency of mast cell detection in the 2 groups (Fisher's exact p=0.13). Figure 3.23. shown below presents graphically the results of mast cell numbers detected in the different tissue layers.

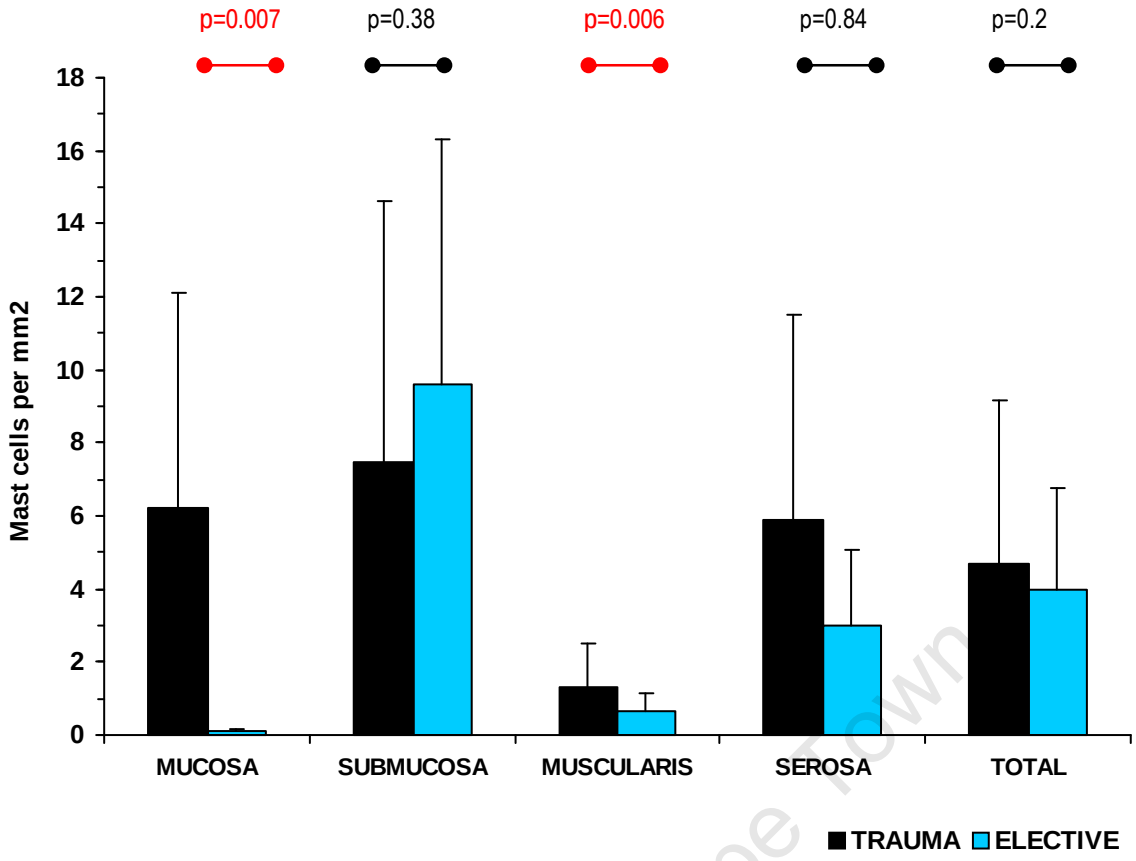


Figure 3.23. Mast cell numbers by tissue layer

Mast cells in the intestinal mucosa

Five of the 8 (62.5%) trauma patients had detectable mast cells within the mucosal layer, whereas only 2 of the 17 elective patients (11.8%). This inter-group frequency difference was found to be statistically significant (Fisher's exact p=0.017). Additionally, trauma patients had significantly higher mean number of mast cells/mm^2 mucosal tissue than did elective patients. Trauma patients had a mean (95% CI) of 6.2 (-0.62-13) mast cells/mm^2 compared with only 0.09 (-0.05-0.24) mast cells/mm^2 in elective patients (p=0.007) (see Figure 3.23.). Plates A and B of the photomicrograph panel (Figure 3.24.) indicate the typical pattern of mast cells seen the mucosa of trauma (A) and elective (B) patients.

Mast cells in the intestinal submucosa

Submucosal mast cells were detected in all 8 (100%) of the trauma patients and in 11 (65%) of the elective patients (Fisher's exact p=0.13). The median (IQR) number of mast cells seen in this tissue layer was 7.5 (3.3-16.9)/mm^2 in trauma and 9.6(0-13.7)/mm^2 in elective patients (p=0.38) (see Figure 3.23.). Mast cells in the submucosa were found to be dark, round cells and were distributed diffusely throughout the tissue, but were generally associated in particular with blood vessels. Photographs of the submucosal mast cell distribution around blood vessels can be seen in Figure 3.24. (C for trauma patients and D for elective patients).

Mast cells in the intestinal muscularis

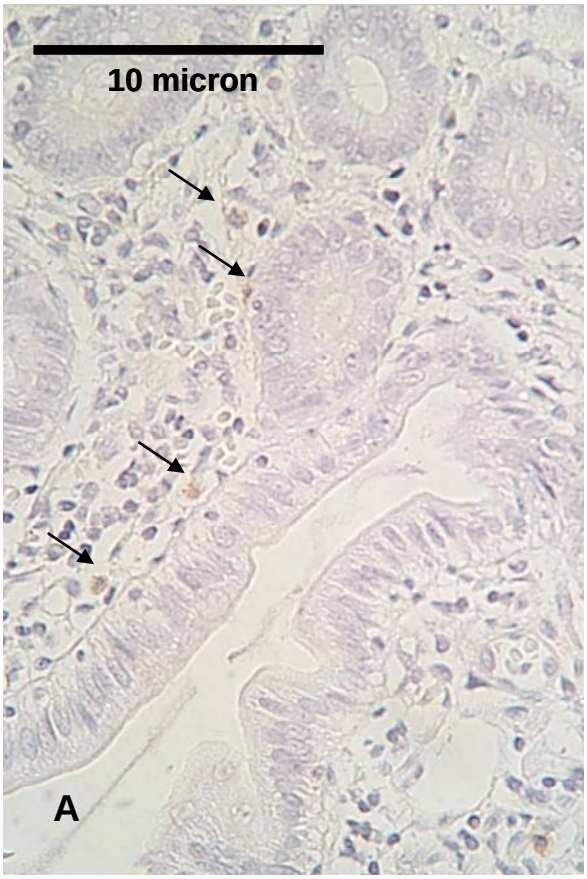
In 7 of 8 (87.5%) of trauma patients and only 7 of 11 (41%) of elective patients, mast cells were seen in mounted sections of the muscularis layer of the human gut (Fisher's exact p=0.03). Additionally, there were significantly more mast cells/mm^2 in trauma cases compared to elective surgery cases – a median (IQR) of 1.3 (0.9-2.8) and 0.66 (0-3.5) respectively (p=0.006) (see Figure 3.23.). Mast cells seen in this tissue layer could be described as paler, elongated cells and were sometimes associated with blood vessels (Figure 3.24. panels E and F).

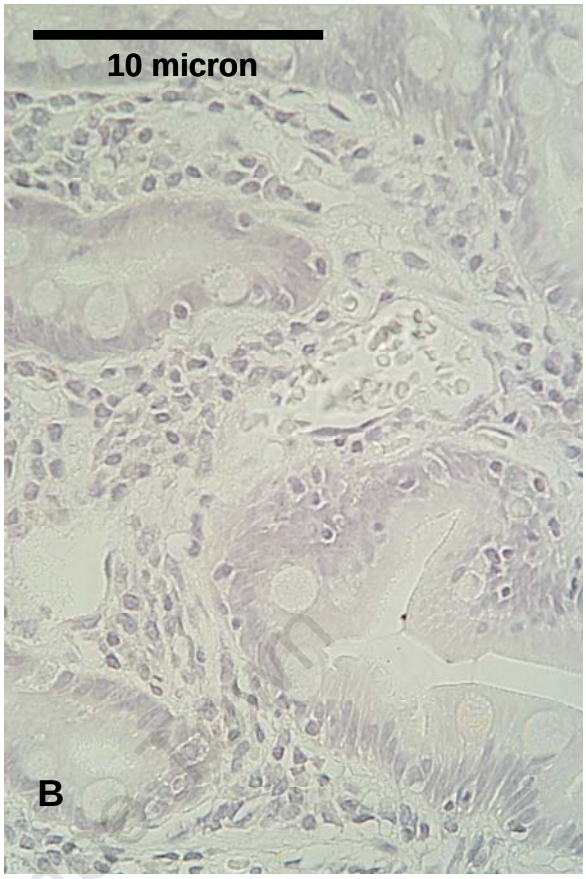
Mast cells in the serosa

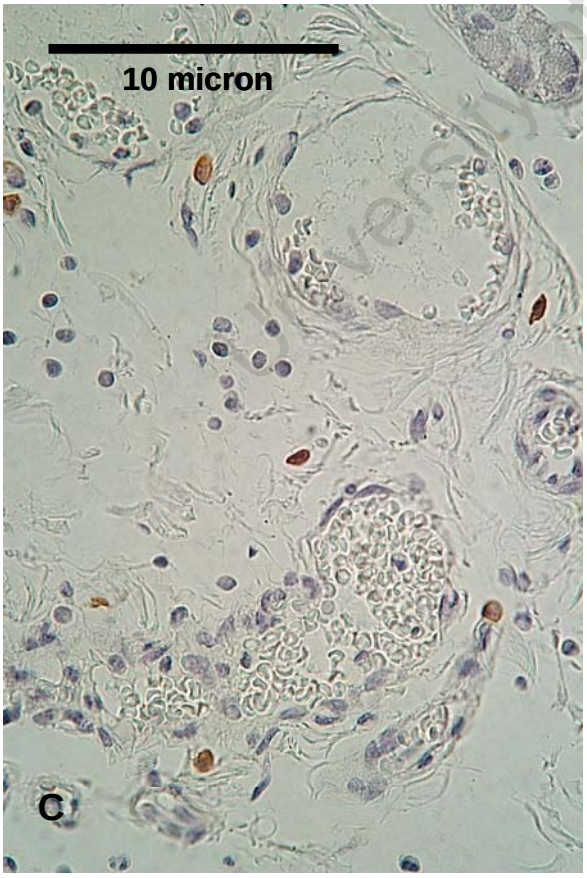
The serosa could not be seen on the mounted sections in 5/8 trauma and 15/17 elective patients. However, in the 3 trauma patients where the serosa could be viewed there was a median of 5.9 (0-0) mast cells/mm^2 (see Figure 3.23.). This is not significantly more than the 3 (0-6.3) mast cells/mm^2 observed in the serosa of the 2 elective patients where this zone could be seen (p= 0.84), given that statistical testing on these low sample sizes must be interpreted cautiously. Figure 3.24. panels G and H present photomicrograph images of mast cells in this zone.

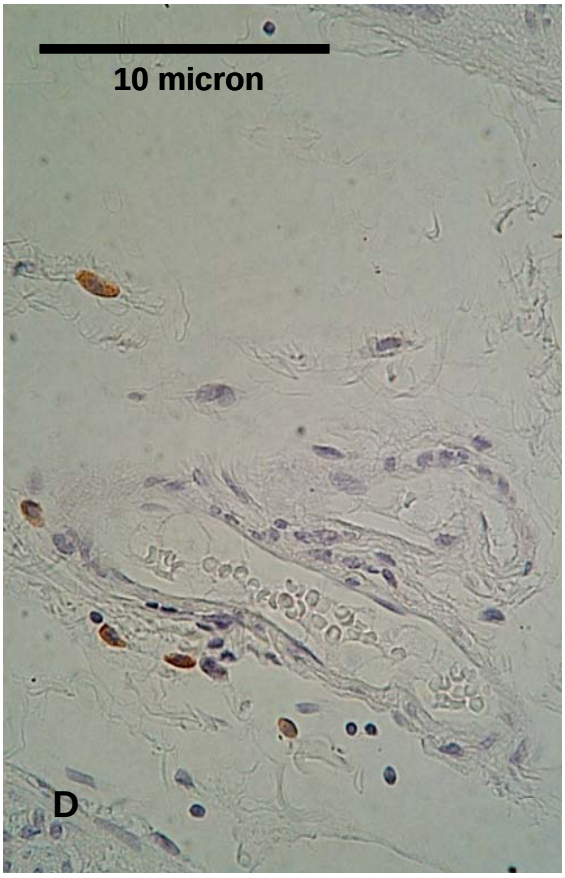
Total mast cells

There was no group difference in the median total mast cells numbers per mm^2 in the entire tissue section – 4.7 (3.1-10.9) in the trauma group vs 3.97 (0-7.4) in the elective group (p=0.2).









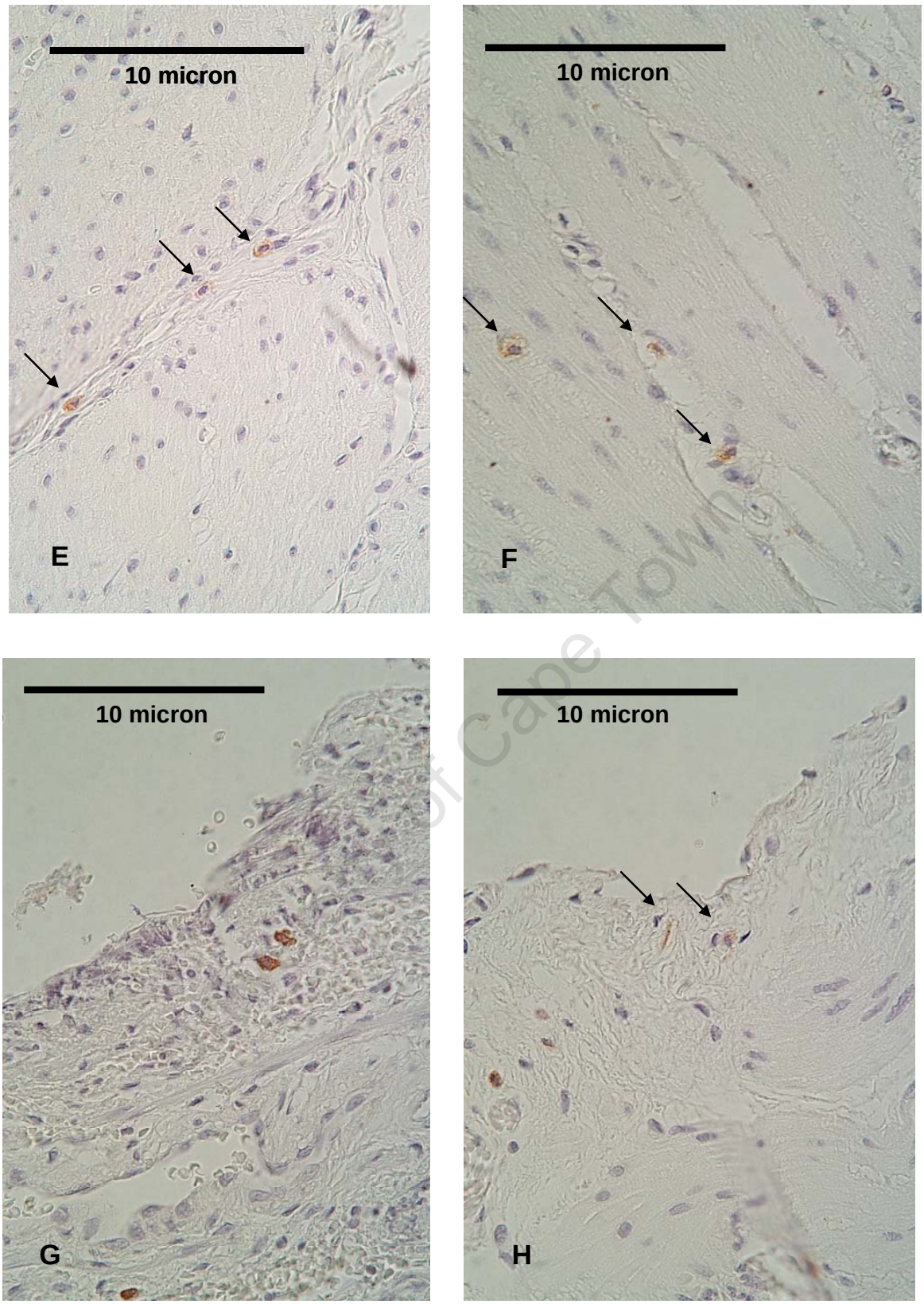


Figure 3.24. Photomicrograph images of mast cells in human gut tissue layers

Figure Legend

A Mucosa of trauma patient indicating pale mast cells (stained brown); B Mucosa of elective patient showing no mast cells; Submucosa of trauma patient (C) and elective patient (D) both with dark mast cells near blood vessels; Muscularis of trauma patient (E) and elective patient (F) with pale mast cells; Serosa of trauma patient (G) and elective patient (H)

In the elective group only, tissue CRF levels were positively associated with the number of mast cells in the mucosa (Spearman's R = 0.49, p=0.06) and in the muscularis (Spearman's R = 0.59, p=0.05), a puzzling result since mast cell numbers in these gut regions were significantly lower than in the trauma group.

In order to interpret the increased mast cells in the trauma group as related to infiltration following a stressful event, mast cell numbers were plotted against the time delay prior to surgery. This is shown in Figure 3.25. below. A small positive trend can be seen suggesting that more mast cells infiltrate the tissue with increasing time from the occurrence of tissue injury.

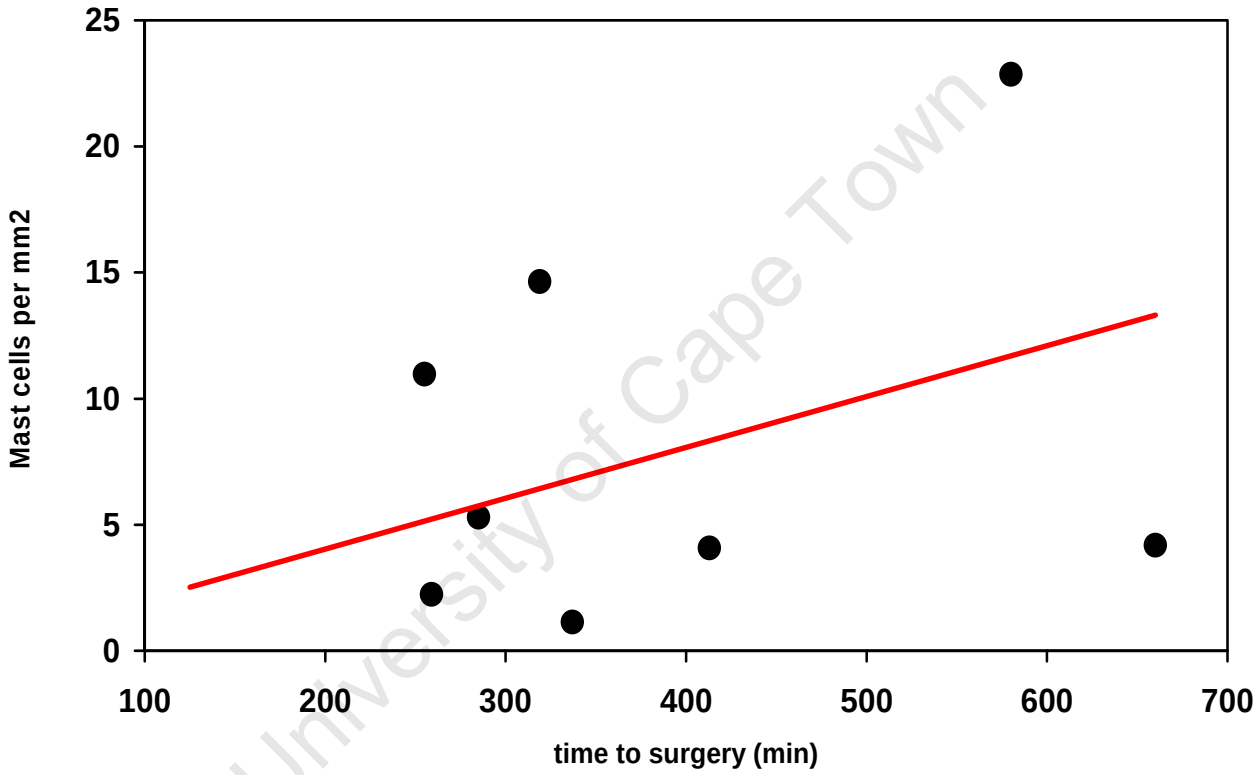


Figure 3.25. Time delay to surgery vs mast cell numbers in trauma cases (n=8)

3.9. Summary

Results from this study indicate that circulating CRF differs between shocked trauma and stable surgical patients. The circulating CRF concentration was statistically higher in trauma patients at all time points and associated with injury severity. The CRF peptide was measured in the intestinal tissue of both trauma and elective patients, with no detectable group difference. Localised expression of CRF could be demonstrated for rat gut. This could not be replicated using stored human gut tissue, although housekeeping genes were successfully amplified for these specimens. Both groups had markedly enhanced intestinal permeability, which was unrelated to tissue CRF levels, but was associated with both extent of hypotension and duration of surgery in the trauma group. In both groups, gastric emptying was retarded, and was significantly slower than both healthy volunteers and

other patient collectives. Indicators of poor gastric emptying were positively associated with circulating CRF levels, but more significantly with the total dosage of morphine administered. Mast cell infiltration was identified on histological examination of the small bowel tissue of both groups. Trauma patients had statistically more mast cells in the mucosa and the muscularis layers of the gut than the elective patients, yet mast cells numbers were only associated with tissue CRF levels in the elective group.

Chapter 4: Discussion

4.1. Introduction

Certain patterns of gut dysfunction including diarrhoea, poor gastric emptying and enhanced intestinal permeability are well recognised phenomena in critically ill patients, especially following circulatory shock (Heyland et al, 1996; Doig et al, 1998; Montejo, 1999). The combination of these conditions makes effective nutritional delivery a challenge and may contribute to poor outcomes in the sickest patients in the hospital (Montejo, 1999; Ritz et al, 2000). Although well recognised, the underlying causes of gut dysfunction in the critically ill have not been well elucidated. The intensive care setting incorporates many factors which are not beneficial for keeping the bowel in good working order. Contributing factors include: the widespread use of opioids, sedatives, and antibiotics; mechanical ventilatory support; and aspects of clinical care, such as peri-operative fasting of patients and the historically enforced supine posture (Kehlet and Moesgaard, 1996; Kölbel et al, 2000; Mutlu et al, 2001; Mutlu et al, 2003). While these various factors almost certainly (individually or combined) play a role in producing gastrointestinal symptoms in ICU patients, they are in fact not the underlying physiological mechanisms that initiate and drive the gut dysfunction so universally observed following shock.

Certainly shock itself is damaging to the gut. In shock, oxygen supply is diverted to the most vital organs, the brain and heart, at the expense of other organs including the gastrointestinal tract. The sequence of insufficient blood circulation, poor gut tissue perfusion, cellular hypoxia, failure of mitochondrial ATP production, acidosis, cellular membrane dysfunction, capillary endothelial damage and eventual cell death disrupts the intrinsic and extrinsic gastrointestinal barriers (Kerner et al, 1995). After resuscitation, tissue ischaemia-reperfusion leads to breakdown in the maintenance of tissue integrity, impairment of intercellular tight junctions, erosions to the mucosal surface and eventual necrotic injury and epithelial cell apoptosis. These effects are worsened by the local release of inflammatory molecules. Even further damage occurs from the effects of free radicals and massive neutrophil activation (Deitch, 1992; Kerner et al, 1995). Gastrointestinal motor, secretory and intestinal barrier permeability functions are all affected. The typical clinical evidence of gastrointestinal dysfunction in the ICU is therefore gastric stasis, diarrhoea and failure to tolerate enteral feeding. Since enteral feeding intolerance is associated with increased morbidity and mortality in the critically ill (Montejo, 1999), the adverse alterations in gut function in this patient group present a serious clinical predicament: - how to successfully deliver enteral nutritional support via an impaired gut. One might think that the obvious suggestion would be instead simply to utilise Total Parenteral Nutrition (TPN). However, while nutritional requirements can certainly be met via this route, poor clinical outcomes seen in some studies may reflect the absence of enteral nutrition, emphasising that the use of the gut is all important.

What is not completely clear is the identity of all the molecules and mediators responsible for inducing these dysfunctional changes to gut physiology. Many substances have been implicated. CRF has emerged as a particular focus of those researching functional bowel disorders and the effects of stress and inflammation on the gastrointestinal tract. CRF is the well-known hypothalamic effector of the hypothalamic physiological stress reaction. Besides its established role as the releasing-factor which drives the HPA axis, more recent data has shown that CRF is of importance in peripheral tissues as well as in the brain. Amassing evidence – particularly, but not exclusively, from animal studies – suggests that CRF is absolutely central to gut-related physiological changes which occur in association with various types of stressors (Taché et al, 2001; Taché and Perdue, 2004; Taché and Bonaz, 2007). The typical bowel dysfunction seen among the critically ill appears to be similar to the CRF-mediated bowel dysfunction associated with stress. The possibility that CRF is integral to critical illness gut dysfunction has not been previously investigated.

Traumatic injury with hypovolaemic shock is a powerful, multi-faceted stressor typified by somewhat unexplained gastrointestinal functional changes evocative of those identified as CRF-related. The broad objective of this study was to investigate whether CRF plays any role in alterations in acute post-operative gastrointestinal function in critically ill adults following traumatic abdominal injury. The specific questions addressed in this thesis are:

1. What is the nature and degree of alterations in acute post-operative gastrointestinal dysfunction in this patient group?
2. Is there an association between circulating plasma CRF and gastrointestinal dysfunction?
3. Is CRF detectable in small intestinal tissue following traumatic abdominal injury, and is it locally produced in the intestine?
4. Is there an association between CRF in small intestinal tissue and gastrointestinal dysfunction?
5. Is there an association between CRF in small intestinal tissue and mast cells; and between mast cells and gastrointestinal dysfunction?

4.2. Study patient groups – The shocked trauma patient versus the elective surgical patient

In order to answer these questions, it was necessary to make an appropriate selection of study subjects. The target group of interest was trauma patients, all of whom had been injured within the previous 6 hours and had an admitting systolic blood pressure of less than 90 mm Hg, or had other evidence of hypo-perfusion. The choice of control group raised some complex problems. The first obvious approach would have been to compare patients with acute traumatic abdominal injury with un-traumatised patients i.e. a comparison between stressed patients and normal, healthy persons. However, this was not possible because the experimental protocol required an invasive surgical procedure. This in itself is a stressor, which would disrupt the controlled design of the study. Additionally, it would be neither surgically nor ethically justifiable to obtain full thickness intestinal tissue from healthy volunteers. Therefore normal healthy individuals could not be included in this

study. Published data from healthy volunteers, however, was utilised in the interpretation of the results of the gut physiology tests which did allow a comparison with a completely unstressed group.

Instead, it was realised that it would be possible to obtain relatively "normal" intestinal tissue from a control group of haemodynamically stable patients undergoing scheduled surgery involving the small bowel, such as in hepato-biliary procedures. These patients were therefore chosen to compare intestinal CRF levels and post-operative bowel function. As surgery itself is a stressor and causes the release of CRF, the elective surgery group were surgically "stressed" in the same way as trauma patients were. This enabled the confounding effect of the stress of anaesthesia and laparotomy on circulating levels of CRF to be controlled for. These elective surgery patients were, however, not shocked. Thus, shock and traumatic gut injury were therefore the distinguishing features of the main study group i.e. abdominal trauma patients. Having controlled for the effect of anaesthesia and laparotomy, which were applied to both patient groups, this allowed for the comparison of CRF measured in macroscopically normal gut tissue from hepato-biliary patients with CRF measured in traumatically injured gut from shocked trauma patients. Additionally, all other main investigational study variables could be compared between groups.

The demographic profile of the two study groups was as follows. The trauma group comprised 8 male, shocked, patients with abdominal gunshot wounds, who had a mean age of 28 years. It is an international phenomenon that trauma patients are typically young males so this was expected to be the case in this study as well. The elective surgery group consisted of 17 stable elective hepato-biliary surgery patients. With a mean age of 50 years, they were statistically older than the trauma patients. This group also included female patients.

Although both study groups were stressed by surgery, the degree of overall stress was different between the groups. This was by design, because the study aimed to detect group differences in levels of CRF, which is itself a marker of stress. In clinical practice, stress can be evaluated using factors such as nature and severity of illness/injury, as well as physiological indicators associated with a stress response, such as white cell count, body temperature and cardiovascular vital signs. Since such an evaluation can be subjective, various systems have been developed to standardise this kind of assessment. This study utilised the Acute Physiology and Chronic Health Evaluation (APACHE) score for this purpose (see Appendix 3). The APACHE score classifies patients' risk of morbidity and mortality based on scored acute physiological variables. More abnormal variables carry a higher score, according to pre-defined categories. Additionally, the APACHE system incorporates the added risk associated with pre-existing chronic diseases (such as diabetes or cancer) as well as advancing age. Again, underlying disease and age both score higher points. APACHE scores have prognostic

value based on the combination of the two elements of the scoring system – the acute physiology variables and the chronic health variables (Knaus et al, 1985).

In this study, the two groups had similar APACHE scores. However, this must be interpreted with the significant inter-group age difference in mind. The increased physiological derangement in the trauma group was compensated for by the increased age of the elective surgery group. This illustrates that it is possible for young patients, such as trauma patients, to obtain high APACHE scores because they present with hugely deranged physiological variables resulting from massive injury. This clinical scenario scores high points despite a lack of chronic health indicators in a young, fit patient group such as this. However, this is usually only true in the immediately post-injury period, because APACHE scores drop as acutely disturbed physiology responds to emergency medical care. In this study, APACHE scores were derived from the data of the first 24 hours in ICU – after resuscitation of the trauma patients. Thus, the scores in the trauma group may appear lower than would be expected considering the injuries sustained. The converse is reflected in the elective surgery group. Since they were older, they generally accumulated more prognostic APACHE points for chronic health variables. Yet, being stable, they did not score very highly for acute physiology elements. Thus, the two groups accumulated APACHE scores in two different ways. Therefore, the APACHE scores do not accurately indicate how much more systemically stressed the trauma patients were in comparison with the elective patients.

For this reason, it is important to look at other indicators of stress that separate the two groups, such as the Injury Severity Score (ISS) – another standardised scoring system. The trauma group had a mean ISS of 26.5. An ISS of >15 is generally accepted to be indicative of serious injury (Baker et al, 1974; Baker and O'Neill, 1976). Therefore, the mean score for the trauma group can be considered a high severity score, and ISS in this range is associated with increased hospital mortality and longer hospital stays (MacKenzie, 1984; Wardrope et al, 1990). The Injury Severity Score can also be used to predict the risk of mortality (Champion et al, 1989). In this study the trauma group had a median predicted risk of mortality of 15%. This is relatively high for trauma patients and confirms their severity of injury.

The degree of shock of the trauma patients is illustrated by the very low mean presenting systolic blood pressure of only 76 mmHg together with the mean requirement of over 11 litres of intravenous fluid in the first 24 hours. This is statistically more than the elective group who, though undergoing rather longer surgical procedures, only required a mean of less than 5.5 litres of IV fluid during the first post-operative day. Therefore, the cardinal distinction between the two groups as intended in the study design had been met.

The difference in degree of stress between the two groups is further demonstrated by the differences in outcomes between the two groups. Length of ICU and hospital stay was longer but not statistically so in trauma patients. Significantly more trauma patients required mechanical ventilation and more time on mechanical ventilation, which was statistically correlated to the severity of injury. This group also needed significantly more surgical re-interventions than elective patients. The trauma group developed more nosocomial infections, as would be expected, although this only approached statistical significance. Therefore, taking all the outcome variables together, the trauma group overall had a trend to worse outcomes than the elective group, which supports the conclusion that they were the sicker and therefore the more stressed group.

4.3. Plasma and tissue CRF

The first aim of this study was to determine circulating CRF levels in the two groups over the first 24 hours following surgery. The results showed that the median plasma CRF concentration was significantly higher in trauma patients compared to stable elective patients at each time point (see Figure 3.2. and Table 3.3). Thus, in the trauma group, median plasma CRF exceeded the reference range by threefold while median CRF concentrations in the elective patients were only marginally above the upper limit of the reference range. The significantly higher plasma CRF concentrations in the trauma group are in line with the recognised role of centrally produced CRF as the principal driver of the stress endocrine response. Since hypotension is a particularly potent stimulus for HPA activation (Zaloga and Marik, 2001; Marik and Zaloga, 2003) it can reasonably be concluded that the combination of severe injury together with shock in the trauma patients resulted in an increased secretion of hypothalamic releasing factors compared with the stable elective surgery patients, as expected. No previous report of plasma CRF levels in trauma or elective patients could be found for comparison.

Trauma patients not only had high plasma CRF levels in the immediate post-injury period, but high plasma CRF levels in this group persisted at the final end-point of measurement for this study, which occurred at 24 hours post-operatively. Interestingly, the plasma CRF concentration at 24 hours post-operatively was correlated with Injury Severity Score. This suggests that plasma CRF is directly related to degree of stress and therefore could be a novel surrogate marker of outcome among trauma patients.

The second investigation carried out was the measurement of CRF in intestinal tissue. The presence of CRF family peptides has previously been shown in rodent gastrointestinal tissues (van Tol et al, 1996; La Fleur et al, 2005; Porcher et al, 2006). Very limited data from studies of human gastrointestinal tissue indicates that CRF peptide is detectable in the normal and inflamed human colonic mucosa using immunocytochemical techniques (Kawahito et al, 1994; Kawahito et al, 1995). These two cited studies comprise the few direct reports of CRF peptide in the human gastrointestinal

tract, but they focus on detection of CRF peptide only in the colon, and in a manner that is not quantitative. The second aim of this study was to investigate and quantify the presence of CRF peptide in human small intestine following traumatic injury to the small bowel.

Using radio-immunoassay, this study showed that CRF peptide is present in small bowel tissue from abdominal trauma patients and elective surgery patients (Figure 3.3.). Results showed that CRF levels in the two groups were similar. Intestinal tissue specimens from the elective surgical group represent the nearest to normal full thickness tissue it is possible to obtain from the human gut in the living being. As far as can be established, this is the first report of CRF peptide in human small intestine, and the first to quantify CRF peptide in "normal" small bowel and following shock and serious penetrating abdominal injury.

On superficial inspection, the tissue levels of CRF appear to much lower than plasma levels. However, a direct comparison of plasma and tissue levels is not valid since they are expressed in different ways. Plasma CRF is expressed as a concentration (pg/ml). Tissue CRF is expressed as a percentage of total tissue protein, accounting for the mass of the tissue specimen originally taken. Even though it is not possible to comment on the levels of CRF in tissue relative to plasma levels, it is still reasonable to make comparisons between tissue samples analysed in the same way. This raises an important question. Assuming that the levels of CRF found in the small intestinal tissue of elective patients represent "normal" basal levels, why do stressed trauma patients not have much higher levels by comparison, especially since plasma levels were so much higher in this group? This expectation is supported by work which has shown that inflammation significantly increases the amount of CRF in human colon tissue compared with baseline levels (Kawahito et al, 1995).

There are four possible explanations for this finding. The first relates to the fact that the abdominal trauma patients in this study were shocked. Therefore, a state of hypo-perfusion and tissue hypoxia of the entire gut existed for some hours prior to obtaining the tissue specimen in the operating theatre. Such a state could result in low levels of tissue CRF because plasma CRF cannot be delivered to under-perfused intestinal tissue. This argument rests on the reasoning that CRF measured in tissue was delivered via the blood circulation. CRF released centrally from the hypothalamus enters the portal venous system of the pituitary from where it can reach the systemic circulation. From the circulation it could then enter tissues, including the intestines. The half-life of CRF circulating in human plasma is approximately 4 minutes (Schürmeyer et al, 1984). This may be too short to result in significant tissue penetration to a relatively ischaemic gut, even when plasma CRF levels are high. Therefore, hypo-perfusion of the gastrointestinal tract as a result of circulatory shock could reduce the amount of CRF delivered to the tissue, and therefore reduce the levels measured in tissue specimens.

The second possible explanation for trauma patients having tissue CRF levels similar to those of elective patients is that the increased production of central CRF was countered by increased utilisation/breakdown of the molecule in the intestinal tissue of the trauma group. The short half-life is one mechanism. Additionally, in tissue damaged by traumatic injury there would be more protease activity which would lower the levels of active CRF.

The third possibility for the relatively low levels of tissue CRF in trauma patients is that the overall amount of CRF peptide present in full thickness specimens is extremely low in proportion to the total amount of protein in the tissue. The method used in this study referenced CRF to total protein in the intestinal specimens, in order to compensate for an increase in bowel oedema following resuscitation. Although the resuscitation fluid was mainly crystalloid, it is possible that the bowel oedema included some albumin from leaky capillaries, which may have reduced the proportional amount of CRF measured. The total amount of peptide detectable may also relate to the histological structure of the gastrointestinal tract, and the potential cellular sources of the CRF peptide. Cellular sources of CRF in the gastrointestinal tract are reportedly enterochromaffin cells, mucosal epithelial cells, and macrophages in human colon (Kawahito et al, 1994), as well as enteric nerve fibres and cell bodies in the submucosal and myenteric plexuses in guinea pig small and large bowel (Liu et al, 2006), rat stomach (Porcher et al, 2006) and rat small intestine (La Fleur et al, 2005). When using a full thickness bowel specimen, it is highly probable that the proportion of cells actually producing CRF relative to the total number of cells of different types, and the amount of muscle protein in any given sample is extremely low. To improve detection, it may be possible to micro-dissect the ganglia, divide the specimen into distinct histological layers, or isolate particular cell populations, in order to detect the amount and source of CRF peptide present in intestinal tissue.

Of the potential cells reported to produce CRF, this study focussed on mast cells because of the established association between CRF-related stress effects and these cells (see Chapter 1 for full review). The study objective was to investigate whether mast cell infiltrates were present in small intestinal tissue of trauma and elective patients, and if there was an association between mast cells and tissue CRF. By analysing histological sections for mast cell infiltration, it was found that these cells occur in all gut layers in both groups. However, in the trauma group significantly more patients had mast cell infiltrates, and the group had significantly more mast cells in the mucosa and muscularis layers compared with elective patients. This finding is in general agreement with published data which reports mast cell infiltration in the mucosal layer of various regions of the human intestine (Siegert et al, 2004; Wang et al, 2007; Wallon et al, 2008). These published studies, however, only report on analysis of superficial tissue specimens obtained during endoscopy and biopsy, while this study has investigated the full thickness intestine. Therefore, it is difficult to deduce the absolute significance of the results of the mast cell distribution in the different gut layers. The higher mast cell numbers in

trauma patients compared to elective patients, however, suggests that mast cell infiltration may be stress-related, as hypothesised.

If this is true, the positive correlation between mast cells and CRF in the elective group but not in the trauma group is an apparently contradictory finding. The probable reason, though, is that the sample size of the trauma group was simply too small to demonstrate such an association. The association between mast cells and tissue CRF in the elective group still supports the hypothesis that CRF and mast cells somehow interact in the human gut. Mast cells have been shown to express CRF receptors, as well as to synthesise the CRF peptide, suggesting that autocrine processes may be present (Kempuraj et al, 2004; Cao et al, 2005; Wallon et al, 2008). Indeed, mast cells can be induced to degranulate by CRF via a CRF-receptor mediated mechanism, and are therefore CRF targets (Theoharides et al, 2004). In vitro work on human gut tissue has shown similar results. Using colonic biopsy specimens from healthy volunteers, Wallon et al (2008) demonstrated in a controlled experiment that exposure of biopsies to CRF resulted in mast cell activation, and that mast cells exclusively expressed both CRF-1 and CRF-2 receptors. The finding of this study therefore provides further indirect support for a CRF-mast cell interaction in the gastrointestinal tract.

The fourth possible explanation for similarly low levels of CRF in both study groups is that CRF is not produced locally in human intestine. It is not possible to discern whether localised production of CRF occurs in intestinal tissue simply by investigation of CRF peptide levels, and therefore this methodology cannot answer this question.

4.3.1. Evidence of CRF synthesis in human gut tissue

The only way to directly measure whether intestinal tissue does synthesise CRF is to measure CRF mRNA expression in intestinal specimens. In order to answer this question, RT-PCR analysis was performed on RNA extracted from intestinal tissue from trauma and elective patients. Using rat tissue to optimise the laboratory technique, CRF mRNA was strongly expressed in both the positive control tissues of rat brain and hypothalamus. Importantly, rat intestinal tissue also expressed significant levels of CRF mRNA. Restriction enzyme digestion and cycle sequencing confirmed the identity of the PCR product as CRF mRNA (see section 3.3 of Chapter 3). Of importance, the expression of CRF in rat gut occurred in tissue specimens from unstressed animals suggesting that basal CRF expression occurs in this species. This finding is in agreement with the work of La Fleur and co-authors (2005) who demonstrated local synthesis of CRF in rat ileum, even under basal conditions. The demonstration of CRF mRNA expression in unstressed rat gut during the optimising phase of this study was encouraging, because it seemed to correspond to the finding of CRF peptide in the stable elective study patients. However, work involving animal models cannot necessarily be directly extrapolated to human patients.

The experimental procedures using human tissue in this study proved to be more challenging. Despite extensive technical effort and repeated experiments (including assistance by an expert), no CRF mRNA was detected in human gut tissue. Why should this be so? The first possible explanation could be related to the size of tissue specimens taken from humans compared to rats. Samples obtained from both species were approximately 50-100mg in weight and roughly $5mm^2$. A specimen of this size represents a tiny segmental section when taken from human gut, but could be on half to two thirds of the entire intestinal circumference of a rat. The rat specimen then could have included anatomical structures, such as nerve ganglia or peri-intestinal tissue conceivably responsible for CRF synthesis, that were not present in the human intestinal samples. That is, the proportion of signal-producing cells per relative circumferential area might be different in rat and human. If this is so, it would make CRF mRNA much easier to detect in rat intestine.

A second possible reason for the failure to detect CRF mRNA in human specimens is that stress alters transcription of CRF in such a way that detection of mRNA becomes especially challenging. Some evidence for this does exist, at least where centrally produced CRF is concerned. For example, it is apparent that under conditions of acute stress, intracellular inhibition of CRF transcription occurs, perhaps by repression of cAMP, which is required for CRF transcription (Shepard et al, 2005). Once CRF has been released, further activation of CRF transcription is required to restore levels of CRF mRNA (Aguilera et al, 2007). Rodent studies have shown basal CRF mRNA expression follows a diurnal pattern (Watts and Swanson, 1989; Lightman et al, 1993). Additionally, transcription of CRF during acute stressful events increases rapidly, but is a transient phenomenon (Kovacs and Sawchenko, 1996; Kovacs, 1998; Ma and Aguilera, 1999a; Ma and Aguilera, 1999b; Kovacs et al, 2000). These cited studies showed that during an acute stressor, CRF transcription increases and then returns to basal levels when the stressor is withdrawn. Similarly, research has shown that CRF transcription in the hypothalamus is transient even when a stress continues to be applied (Shepard et al, 2005).

It is unknown whether similar mechanisms impact upon CRF transcription in peripheral organs. If, as suggested by Shepard's results (2005), the stress-induced rise and fall in CRF expression occurs within a period of minutes, the peak of CRF mRNA expression in the tissue of trauma patients in this study may have been missed. The mean delay until arrival in hospital was just over 3 hours, with a further mean 3.5 hours delay before laparotomy commenced. By this stage patients had been stabilised and at least partially resuscitated. At the time tissue was finally obtained during surgery, CRF expression levels in intestinal tissue could conceivably have returned to basal amounts, which may have been undetectable by the molecular methods used here.

Indeed, other groups have also found it challenging to detect CRF mRNA in tissue specimens from various peripheral organs using RT-PCR, even when immunohistostaining techniques for CRF have

been positive, or when CRF-receptors have been detected (Baigent et al, 2000; Klimaviciute et al, 2006). This may reflect the difficulty of working with whole tissue specimens. Alternative methods, such as in situ hybridisation, may be required. For example, CRF mRNA has been shown in normal human colon and in human tissue biopsy from patients with ulcerative colitis using in situ hybridisation (Kawahito et al, 1995). Kawahito's group has also shown CRF mRNA expression in human colon using RT-PCR. The technical differences between their study and this one, is that they used only the mucosal layer of the colon which may respond better to homogenisation. Perhaps even more importantly, they combined RNA extracted from several biopsies before performing RT-PCR. By doing this, a low mRNA signal is hugely amplified.

This study did not produce data to support the concept of localised production of CRF in the gut of traumatically injured patients, or elective surgical controls using full thickness specimens from the small intestine. Thus, in contrast to results from studies of human colonic tissue, CRF mRNA expression in the human small intestine was not found, either under basal or stress conditions.

Finally, the lack of evidence of CRF mRNA in human small intestine may be on a purely technical basis. This will be discussed in a subsequent section of this chapter.

4.4. Gastrointestinal function – what physiological dysfunctions occur?

The next objective of the study was to investigate and compare gastrointestinal physiology in the trauma and elective groups in the immediate post-operative period, and relate it to CRF concentrations. Two markers of gastrointestinal function were measured at 24 hours post-operatively:
- gastric emptying time and small intestinal permeability.

4.4.1. Gastric emptying

Gastric emptying was used as a measure of upper GIT motility. The key parameter for calculating gastric emptying is the area under paracetamol concentration versus time curve, which is abbreviated as AUC with a subscript indicating the time in minutes at which it is calculated. Additionally, both the maximum serum paracetamol concentration reached during the test period (C_{max}), and the time at which C_{max} occurs (T_{max}) are also meaningful when evaluating rate of gastric emptying. The AUC_{120} in shocked trauma patients was significantly lower than that of the elective group. The inter-group difference in the AUC_{150} also approached significance, being lower in trauma patients. This shows that trauma patients had even slower gastric emptying than elective patients. In fact, in the trauma group, paracetamol concentrations remained statistically similar to baseline levels throughout the test, indicating that virtually no paracetamol left the stomach during the 3 hour test period (Figure 3.20.). This was different from the elective group who showed a statistically significant change in paracetamol concentration compared with baseline levels in the 90 minute sample and all subsequent samples. In the trauma group there was a statistical association between AUC_{180} and length of ICU

stay, suggesting that worse outcomes may occur for those patients with delayed gastric emptying. Thus, both the trauma and elective surgery groups had slow gastric emptying times. Compared to previously published results both groups had gastric emptying that was 3-4 times slower than both healthy volunteers (who presumably represent "normal" values) and other patient sub-groups (see Table 3.8).

The two groups had a similarly low C_{max}. Trauma patients had a median C_{max} of only 2.86 ug/ml – approximately half that of the elective patients median value of 4.59 ug/ml, although this difference was not statistically significant. Furthermore, C_{max} values in trauma patients were found to be only 25% that of published values for healthy persons and other surgical groups (Table 3.8.), while in elective patients the C_{max} was less than half of these reported values. These differences between the data from the study patients and published data were statistically significant, confirming the significant impairment in upper gut motility in the study groups. Following the same pattern, the final indicator of gastric emptying time – the T_{max} - indicated a similar, pronounced delay in gastric emptying in both study groups. Again, both trauma and elective patients had T_{max} values more than twice as long as healthy subjects.

Thus, by all parameters study patients displayed markedly decreased gastric emptying. The next objective was to investigate any possible association between measures of gastric emptying and CRF. While trends were seen for an association between high baseline plasma CRF levels and delayed gastric emptying in the trauma group, it was among elective patients that the negative association between AUC_{120} and concurrent plasma CRF concentration reached significance. These results are in line with the study hypothesis that centrally released CRF is associated with poor gastric motile function, and with a multitude of literature which evidences this also (Lee and Sarna, 1997; Chen et al, 2002; Taché et al, 2001; Martinez et al, 2004; Czimmer et al, 2006). Conversely, stimulatory effects of CRF on the lower gut have been shown to increase colonic motor responses i.e. more rapid transit time, enhanced motility and increased defaecation (Williams et al, 1987; Mönnikes et al, 1993; Martinez and Taché, 2001; Martinez et al, 2002). Clinically, this presents as diarrhoea. However, results of this study show an extremely low incidence of diarrhoea during the first 3 days post-operatively, which was unrelated to plasma CRF levels in either study group. This could be related to use of systemic morphine in the acute post-operative period, which is known to inhibit gastrointestinal peristalsis (Bueno and Fioramonti, 1988).

Also of interest was the association found between shock and retarded gastric emptying. In the two study groups combined, there was a significant and strong negative correlation between the T_{max} and minimum systolic blood pressure in the first 24 hours ($R=-0.73$, $p=0.0003$). The meaning of this result is that lower systolic blood pressure was associated with increased delays in gastric emptying. This finding clearly supports the link between shock and poor gut function, as hypothesised in this study.

Shock and ileus-associated metabolic parameters such as acidosis and gut hypoxia-reperfusion together with localised intestinal tissue damage are probable factors involved. Indeed, the poor gastric emptying observed in the current research is very much in line with the abnormalities in gastric emptying which are known to affect various sub-groups of ICU patients especially following shock (Ott et al, 1991; Kao et al, 1998; Nguyen et al, 2007; Landzinski et al, 2008). In the current study there was no association, however, between poor gastric emptying and tissue CRF levels. Post-operative ileus is known to affect mainly gastric and colonic motility though, while the small bowel continues active, if not entirely nomal, peristalsis (Tournadre et al, 2001; Miedema et al, 2002). Since CRF was measured in intestinal tissue and not gastric tissue, this is a possible reason that no association was found.

By interesting contrast with the trauma study patients, patients having undergone abdominal aortic aneurysm (AAA) repair reportedly have normal gastric emptying within 18 hours of the surgical procedure (Avrahami et al, 1999). Like the trauma group in this study who were haemodynamically shocked, patients undergoing major vascular surgery may also experience a period of bowel ischaemia due to atherosclerotic disease, hypotension, or aortic cross-clamping followed by reperfusion of the gastrointestinal tract. Yet, as reported by Avrahami et al (1999) normal gastric emptying (defined as an AUC_{60} of >600 mg/min/l) returned within the first post-operative day. It is therefore possible that the extent of direct injury to the bowel itself was an additional factor contributing to the significantly slower gastric emptying time observed in the trauma group (mean AUC_{60} of only 72 µg/ml/min).

Besides the shock and bowel injury, other factors are likely to have contributed to the impaired gastric emptying observed in this study. Such factors include: the experience of open major surgery itself; the associated manipulation of the intestines; pre- and post-operative pain; and exposure to various anaesthetic and analgesic drugs. All of these factors are well known and virtually inevitable causes of gastric motor stasis in the post-operative period (Kehlet, 1997; Mattei and Rombeau, 2006).

The results from this study reveal a strong link between morphine dosage and poor gastric emptying. In the two groups combined, the total morphine dose administered via all routes combined was negatively correlated with AUC and C_{max} for paracetamol. This is consistent with the known inhibitory effect of morphine in particular on the gastrointestinal tract (Nimmo et al, 1975; Minami and McCallum, 1984; Milligan et al, 1988; Rowbotham et al, 1988; Yee et al, 1991;Petring et al, 1995).

This inhibitory effect of morphine on gastrointestinal motility is reportedly dependent to some extent on the route of delivery and not simply on the total dosage of this analgesic, however. This is certainly supported by some of the other study findings related to analgesia and gastric emptying. Trauma patients all received an intravenous morphine infusion for post-operative analgesia during

the first 24 to 48 hours. The elective surgery patients on the other hand mainly received epidural analgesia using a combination of 0.01% morphine and 0.1% bupivacaine. Both groups, however, had access to rescue analgesia in the form of bolus intravenous morphine. The trauma group received a significantly (i.e. four times) higher total morphine dose via all routes combined than elective patients, yet the total dose of morphine in the trauma group alone was not correlated with poor gastric emptying. Neither was there an association between the dose of morphine delivered only via the epidural route and retarded gastric emptying – perhaps because of the fractional dose required for good pain control when using peridural forms of analgesia. However, the total morphine dose administered systemically was indeed negatively correlated with C_{max} and AUC in the two study groups combined. Taken together, these three results imply that it is indeed not merely the morphine dose, but also the route of administration of morphine that impacts upon gastric emptying.

These results support the generally accepted view (based on widely and systematically reviewed evidence) that epidural analgesia per se has less of an impact on gastrointestinal function than other routes of analgesic delivery, especially when opioids are used (Moniche et al, 1995; Kehlet and Moesgaard, 1996; Steinbrook, 1998; Liu and Wu, 2007). That is, epidural opioids alone have less of an inhibitory effect on gastrointestinal motility than intravenous opioids alone (Morimoto et al, 1995; Barzoi et al, 2000). By meta-analysis, epidural local anaesthetic-opioid combinations have actually been found to be superior to opioid-only epidural analgesia in this regard, probably because the sympathetic blockade achieved by using epidural local anaesthetics effectively increases gut motility (Liu and Wu, 2007). Thus, the use of the local anaesthetic, bupivacaine, together with epidural morphine could explain why the elective patients had faster gastric emptying that the trauma patients.

4.4.2. Intestinal Permeability

The second marker of gut function used in this study was small intestinal permeability. Results showed that both trauma and elective surgery patients demonstrated greatly enhanced intestinal permeability, at levels 15-30 times that of healthy volunteers – in both cases a statistically significant increase. Further, 71% of trauma patients and 85% of elective patients had urinary sugar ratios in the range considered “highly increased permeability” (Faries et al, 1998). There was no inter-group difference in the lactulose to mannitol ratio measured at 24 hours post-operatively, and no association with plasma or tissue CRF in either group. However, group differences did emerge in three findings. Firstly, in the trauma group circulatory shock (represented by minimum systolic blood pressure) was significantly and strongly negatively correlated with lactulose:mannitol, indicating that worse shock was associated with excess permeation of lactulose. This was not seen in the stable elective surgery patients. Secondly, the volume of intravenous fluid was strongly and significantly correlated with urinary lactulose concentration in the trauma group but not in elective patients. Thirdly, lactulose:mannitol was positively and significantly associated with duration of surgery in the trauma

group but not the elective group, suggesting that intestinal permeability may be related to handling of the bowel during the operative procedure.

These three findings are of clinical interest since they are related to aspects of patient management. The first correlation – that of shock with lactulose permeation - is consistent with the previously discussed association between shock and delayed gastric emptying, and further supports the recognised clinical connection between shock and poor gastrointestinal function. Shock might cause mucosal hyper-permeability in a number of ways (see Chapter 1 for full review). Shock-related under-perfusion of the gut may result in failure to meet the metabolic demands of the gut tissue, and the consequences could include mucus layer disruption, epithelial cell apoptosis, and villous loss (Rombeau and Takala, 1997; Minor and Isselhard, 1998; Holland et al, 2005; Rupani et al, 2007), as well as reduced expression of membrane-associated proteins such as occludin and zona occludens protein-1, which are crucial regulators of intestinal tight junctions (Han et al, 2002; Constantini et al, 2008). Shock-induced barrier impairment also appears to hinge upon augmented release of inflammatory cytokines such as TNF-α and IL-6 (McKay and Baird, 1999; Spindler-Vesel et al, 2006).

Ischaemia-reperfusion may be an additional mechanism whereby shock causes intestinal hyper-permeability. This mechanism deserves further discussion. Patients included in the trauma group were by definition shocked and therefore tissue ischaemia is a reasonable physiological collateral under this circumstance. Besides the resultant local production of free radicals and cytokines that occurs in low flow events, as has been mentioned above, reperfusion itself may be damaging to the gut. Loss of intestinal barrier function is associated with severe morphological changes to the small intestine, opening of tight junctions and epithelial cell necrosis and apoptosis. These structural changes in turn seem to be correlated with the severity and timing of the reperfusion injury (Shah et al, 1997; Sun et al, 1998; Noda et al, 1998; Hotchkiss et al, 1999; Hotchkiss et al, 2000; Ikeda et al, 2002; Zhang et al, 2002; Chang et al, 2005; Higuchi et al, 2008).

The injury that results from reperfusion may be at least partially dependent on aspects of resuscitation. For example, it has been reported that the type of resuscitation fluid used in shock may have a critical influence on gut barrier function. Volume resuscitation with crystalloid solutions appears to result in a worse gut barrier defect, more bowel oedema, more pronounced gut histological damage and more severe lung injury than resuscitation with colloids (Shi et al, 2002; Vega et al, 2008). This may at least somewhat explain why the second correlation - between the volume of intravenous fluid received in the first 24 hours post-injury and the urinary appearance of lactulose - was found in the trauma group and not in the elective group. The trauma group had received a significantly larger volume of crystalloid fluids in the first 24 hours than the elective surgery group. Of course fluid volume is also a proxy marker for degree of shock, and the relevance of shock to intestinal permeability has been discussed above.

Thus, the two abovementioned correlations can be understood to relate broadly to shock and gut ischaemia – both known to be associated with intestinal hyper-permeability. This explains why shocked trauma patients should demonstrate profound hyper-permeability. However, one cannot dismiss the fact that the elective surgery patients were not shocked, did not experience gut ischaemia, and did not require resuscitation, yet were also found to have significant intestinal hyper-permeability. Why should this be so? Was it merely due to the induction of anaesthesia, intraoperative fuids and effects of abdominal surgery? Many of these patients had bile duct obstruction and were therefore clinically jaundiced. There is considerable evidence from in vitro studies as well as trials involving animals, that jaundice itself may be associated with various disturbances in gut barrier function (Deitch et al, 1990; Parks et al, 1996; Reynolds et al, 1996; Karsten et al, 1998; Kordzaya and Goderdzishvili, 2000; Parks et al; 2000; Ogata et al, 2003; Gatt et al, 2007). Studies involving human subjects are limited (Sedman et al, 1994; Welsh et al, 1998; Kuzu et al, 1999). Despite the limited data in man, jaundice is widely believed to promote intestinal permeability, and the absence of bile from the gut lumen is thought to unfavourably impact on intestinal microflora populations and permeation. Data from the elective surgical group certainly supports this belief, since their lactulose:mannitol ratios were up to 45 times that of healthy volunteers (Table 3.6). Many of the elective surgery patients had malignant disease of the pancreas or biliary system, which also could have resulted in increased inflammatory mediators and pre-operative malnutrition – both risks for enhanced permeability (Deitch, 1992; Ferraris and Carey, 2000).

Finally, coming back to the third clinically important correlation related to intestinal permeability: - an association between the lactulose-mannitol ratio and duration of surgery was found in the trauma group but not the elective group. There are two likely interpretations of this result. Presumably when the traumatic injury was more extensive (and therefore risk for hyper-permeability was higher), surgery simply took longer. Alternatively, trauma patients endured extensive intestinal handling through the duration of the operation which negatively affected intestinal barrier function.

Surgical manipulation of the bowels is a known causative factor in permeability changes to the small intestinal mucosa. Handling of the intestines during surgical procedures in animal models and humans has been shown to induce oxidative stress, alterations to the mucosal membrane structure and function, and increase intestinal permeability (Anup et al, 1999; Prabhu et al, 2000; Anup et al, 2001; Thomas et al, 2001; Reddy et al, 2005). The elective group actually underwent slightly longer surgical procedures, yet did not demonstrate the same correlation. This can be explained by the difference in the surgical approaches to a trauma laparotomy and a hepato-biliary procedure. In gunshot wounds to the abdomen the bullet frequently passes through multiple loops of bowel. The surgeon is therefore obliged to visually inspect the entire length of bowel to find and repair each wound. In order to do this, significant and sometimes repeated handling of the bowel is required. In

hepato-biliary procedures time is taken in careful mobilisation and dissection of the biliary tract, pancreas and liver, while the small intestinal bypass loop is only created at the end of the procedure and is usually performed fairly quickly.

The level of defective permeability that represents a pathological impairment has not been definitively established for the critically ill, who are only a sub-group of all patients in which the concept of defective gut barrier function is clinically relevant. The clinical significance of increased gut permeability to large sugar molecules is not entirely known, but it is thought that it may be associated with the absorption of toxins such as lipopolysaccharide derived from luminal flora (Deitch et al, 1996; Moore, 1999). Critically ill patients may develop enhanced intestinal permeability via different mechanisms, and more probably via the cumulative effect of various mechanisms combined. Results from this study certainly support this view.

A mast cell-driven mechanism may also be implicated in increased intestinal permeability. An objective of this study was to investigate the possibility that mast cells play a pivotal role in critical illness related gut dysfunction, especially in barrier dysfunction. The finding of increased mast cell numbers which correlated with tissue CRF has already been discussed. This result, which links together mast cells and CRF in human small bowel, is relevant to intestinal permeability in the following way. Mast cells have CRF receptors, and degranulate when stimulated by exposure to CRF (Theoharides et al, 2004). Their secretory products (such as cytokines and histamine) are inflammatory and - in the gut - result in disturbances in mucosal barrier and transport physiology, which can be blocked by both CRF antagonists and mast cell stabilisers (Santos et al, 1999; Barreau et al, 2007; Santos et al, 2008). Additionally, mast cell knock-out animals do not display intestinal hyper-permeability in response to CRF, indicating that both mast cells and CRF are crucial for the response (Teitelbaum et al, 2008).

While the findings of this study support the general link between tissue CRF and mast cell numbers, a connection between this association and intestinal permeability was not specifically shown. In vitro experiments on normal human colonic biopsies showed that exposure of biopsies to CRF resulted in mast cell activation and increased permeation of large molecules, that CRF antagonists inhibited or abolished the hyper-permeability response to CRF, and that mast cells exclusively expressed both CRF-1 and CRF-2 receptors (Wallon et al, 2008). Interestingly, as with the data from this study, they did not show a related increase in *paracellular* permeability as has been shown in previous studies (Crowe and Perdue, 1993; Barreau et al, 2007). This may indicate that in the human gut, mast cell-CRF interaction increases permeability via transcellular rather than paracellular routes.

Comparisons between Wallon's work on human gut tissue specimens and this thesis should be made with caution, however. Biopsy specimens obtained during endoscopy are superficial samples of

tissue, and healthy volunteers by definition have no pathology. In the current study, full thickness gut specimens were obtained from patients who had undergone severe traumatic injury. This study also investigated small intestine while Wallon used colon, and there are important functional differences between these gut regions. It is very difficult to know to what extent *in vitro* work can be extrapolated to the extreme complexities of the human gut in the living person. Nevertheless, the work of Wallon et al (2008) is new evidence of the involvement of CRF acting in a peripheral organ via mast cells to regulate barrier integrity in the human gut.

4.5. Technical matters – detection of CRF mRNA

This study found that CRF mRNA is either not expressed in human small bowel tissue, or that it was not detected either because of technical factors or because of very low levels of expression. The first consideration is whether the techniques were optimal for CRF mRNA detection. Extensive optimising of the methods and procedures was carried out using positive control tissue known to express CRF. Since human hypothalamus or brain tissue was not available, rat hypothalamus and brain tissue was used as a positive control. The PCR primers were therefore carefully selected to enable amplification of homologous regions of human and rat cDNAs. There was 100% homology for the primers and the region between the primers had 87% homology.

Very extensive work was done to optimise the RT-PCR protocol, taking into account tissue harvesting and storage, RNA extraction and PCR conditions such as temperature and buffer concentration. Having optimised these various conditions using rat tissue, CRF mRNA was successfully amplified in rat brain, hypothalamus and gut (see section 3.3.1). The product was confirmed to be CRF by restriction enzyme digestion and sequencing (Figures 3.11, Figure 3.12. and Table 3.4.). Following the same optimising steps as had been done for rat tissue, RT-PCR was then carried out on human gut specimens. Despite exhaustive attempts as listed in the results, CRF mRNA was not detected. The question is why not?

The first possible problem was the poor quality of RNA extracted from the study specimens from trauma and elective patients, which had been accumulated and stored for batch analysis (Figure 3.13.). Correct preservation and storage techniques are critical to ensure that endogenous RNAses are not activated. Instantaneous flash-freezing should immediately follow harvesting of tissue specimens. This was the method used in this study with the average time from surgical excision to flash-freezing being only a few minutes. For this method to be completely successful, however, there are two necessary considerations. Firstly, samples must be small enough to fully freeze instantly upon immersion in liquid nitrogen. The samples obtained from elective patients were very small (<50mg), but those from trauma patients were sometimes large and had to be manually dissected apart or divided prior to flash-freezing. Nevertheless, this process was done using sterilised and correctly treated (i.e. with DEPC) tools and materials, and was performed extremely quickly in the

operating theatre. The second point is that once frozen through, tissues must remain frozen at ultra-low temperatures (-80°C) and never be allowed to thaw. While the specimens did not experience any known thaw, even brief periods of thawing can be sufficient to cause RNA degradation and loss. In this study, every effort was made to protect the tissue specimens at each step of the harvesting and storage procedure.

In order to establish whether the storage did affect RNA quality, a fresh specimen was obtained from an elective patient. The specimen was homogenised immediately and RNA was extracted without ever subjecting the tissue to storage. Furthermore, different methods of homogenisation were utilised to maximise the RNA yield. This was done because tissues rich both in protein and fibrous tissue material are difficult to isolate total RNA from, and the overall yield can therefore be low. Cultured cells can be homogenised very easily by vortexing or other agitation, but animal tissues require rigorous disruption of cells in order to extract RNA, especially with low cell density tissue. Yet, excessive force during this step can result in shear damage to the RNA. Homogenisation using a Polytron® was found to be a successful method of breaking up the tissue prior to RNA extraction. Using this method, RNA of good quality was extracted from fresh human gut tissue, but tissue that had been stored continued to produce apparently sub-optimal RNA (Figure 3.13).

Since the demonstration of CRF mRNA in human gut was so critically important to the study, the RNA quality from both fresh and stored human gut was further investigated. This was done by testing the amplification of housekeeping genes to determine whether the RNA from stored specimens was as degraded as it had appeared on the agarose gel. Two genes were tested: - one highly prevalent (GAPDH) and one less prevalent (GUS1B). Results from this PCR analysis revealed that both fresh and, to a lesser extent, stored human gut samples expressed GAPDH as well as GUS1B (Figure 3.17. and Figure 3.18.). Thus, the RNA was of sufficient quality to detect the expression even of lower prevalence housekeeping genes such as GUS1B, even though stored specimens showed apparently degraded RNA on gel electrophoresis. Therefore, poor RNA quality is an unlikely explanation for the failure to detect CRF mRNA in human gut.

Another possible explanation for the failure to detect CRF mRNA may be related to the prevalence (number of transcript copies) and the limits of detection. The technicalities of working with whole tissues mean that the signal being sought is at a very low volume. The argument put forward for the low levels of CRF peptide in gut tissue also apply and must therefore be reiterated. That is, all the possible cellular sources of CRF in the gut are not yet known. Neither are the precise triggers of local synthesis. Therefore, when using a full thickness bowel specimen, one is seeking a signal which might be below the limit of detection. When one is targeting a specific subset of mRNA within the total intact RNA recovered, this problem is magnified.

It is not possible to conclude with absolute conviction that CRF is not expressed in human small intestine, although the results certainly suggest that this is the case. The accumulation of suboptimal technical conditions might have contributed to detection problems since storage was shown to affect the RNA integrity. However, CRF expression was not shown in fresh tissue where RNA was of very good quality. The PCR technique may be unable to detect minute levels of CRF expression if they occur.

4.6. Limitations

This study was constrained by a very small sample size. Despite the use of a busy trauma centre together with a dedicated primary researcher and hands-on involvement in an integrated enrolment mechanism, recruitment was empirically and pragmatically much more challenging than could have been anticipated. A surprising number of trauma patients did not meet pre-set criteria for shock or for the required time window to presentation, probably because the hospital centre is a tertiary care facility and receives trauma patients who are, more often than not, stabilised and provided with advanced life support in the field and at other lower level centres prior to transfer. Additionally, post-operative clinical factors (such as a particular surgical manoeuvre e.g. gastro-enterostomy, excess nasogastric drainage, lack of venous access etc) in some cases caused physiological testing to become impossible and further reduced the group size for selected trial procedures. Although powered to detect differences in CRF levels, which was successfully done, the small sample size made meaningful correlations difficult to achieve. This is particularly true when clinical measures are used, and may explain why correlations with CRF were only apparent in the elective group since it was the larger of the two. In the physiological response to stress and surgery, it was found that the two groups more similar than different, and thus the impact of the inter-group differences in CRF on gastrointestinal physiology could not be distilled.

There were some limitations to the control group used as well. Firstly, patients undergoing such major (albeit elective) surgeries cannot be considered completely unstressed, and therefore there is no comparison of a stressed versus an unstressed population in this study. Secondly, the inclusion of elective patients having undergone gastro-enterostomy as part of their surgical treatment clearly poses a problem in the interpretation of gastric emptying tests. Such patients had to be excluded from the analysis of those results, again reducing the effective sample size. Thirdly, it is general practice in the hospital centre used, that most elective surgical patients who enter ICU following this type of major surgery are managed using epidural analgesia. Trauma patients entering ICU are managed using intravenous morphine infusion during the first days of care. This difference in clinical approach to analgesia is relevant when interpreting inter-group differences in gastrointestinal responses. The epidural route of analgesia delivery does not have the same inhibitory effects on gut function as intravenous opiates are known to have (Moniche et al, 1995; Kehlet and Moesgaard, 1996). Fourthly, the inclusion of elective patients with clinical jaundice, and therefore bilirubinaemia was less than

ideal. Finally, the sample of intestinal tissue obtained for this study was very small. It is unclear how representative a small specimen would be of the entire small intestine. These limitations do pose challenges to the interpretation of certain data pertaining to gastrointestinal function that has been presented in this study.

Lastly, on a point of ethics, the main reason for failure to enrol elective patients into the study was lack of informed consent to participate. Trauma patients usually did not have an opportunity to consent prior to sampling, as was approved by the institutional ethics committee (see section 2.3.2.). They were all given the option to later give their free consent, or withdraw and have their data destroyed (which happened in a small number of cases). This difference in the timing of informed consent between the two study groups could be construed as a bias associated with enrolment.

4.7. Summary

This work has for the first time demonstrated the presence of CRF peptide in human gut following traumatic injury. Indeed, as far as can be established, this is the first report of quantitative levels of plasma and intestinal tissue CRF in the critically ill, and the first attempt to link critical illness intestinal dysfunction with locally released CRF in the gastrointestinal tract. Nevertheless, evidence of local synthesis of the CRF peptide in human gut tissue was not obtained despite extensive efforts. This is in contrast to basal expression levels shown in tissue from rat intestine. Both elective and trauma patients displayed significantly abnormal gastric emptying and intestinal permeability together with demonstrable mast cell infiltration of small intestinal tissue layers. These effects were overall more marked in trauma patients and were related to indicators of shock, intestinal manipulation during surgery, the volume of resuscitation fluid and the use of high doses of morphine, particularly via the intravenous route. Additionally, poor gastric emptying in trauma patients was associated with a longer ICU stay suggesting that clinical outcomes may be worse when gastric stasis is present. Gastric dysmotility and intestinal barrier dysfunction were found together with mast cellularity in the human small intestine. Although plasma CRF was correlated with poor gastric emptying and tissue CRF was associated with mast cell infiltration in the elective group, locally expressed CRF was not shown to contribute to the bowel dysfunction associated with traumatic stress and shock.

4.8. Conclusions

Corticotropin-releasing factor is present in human small intestine during elective surgery and emergency surgery for haemorrhagic shock. Plasma CRF levels are increased by surgery, but trauma with haemorrhagic shock has a significantly greater effect on circulating CRF than elective surgery. While mRNA for CRF is detectable in rats, this study did not show CRF synthesis in human small intestine. Mast cell infiltration of the small intestine is increased by haemorrhagic shock. Both elective and emergency gastrointestinal surgery has a marked effect on gastric emptying and intestinal

permeability. The role of peripheral CRF the gastrointestinal dysfunction seen in critically ill patients remains uncertain.

4.9. Beyond the study hypothesis – future directions

The hypothesis that locally expressed CRF contributes to bowel dysfunction following shock and traumatic stress is not supported by this small study. The study found no evidence that peripheral CRF is synthesised in the human small intestine. However, the finding of the CRF peptide together with increased mast cells in the gut of patients following surgery and traumatic abdominal injury indicates that previously proposed CRF-driven mechanisms may somehow be involved. This is a possibility that should be pursued through further research. Further study should also give attention to the potential role of cytokines and glucocorticoids as intermediary molecules in CRF-mediated gastrointestinal effects, as well as the specific location of receptors for CRF and other stress molecules such as glucocorticoids in the human gut.

Relatively little investigation has gone into the mechanism by which gastrointestinal function is disrupted in trauma patients in the ICU. Where threat to life exists, ethical and practical issues may result in other aspects of critical care being prioritised for research. However, one must argue for continued attention to gastrointestinal function in the critically ill, particularly since this study has demonstrated the negative effect of aspects of clinical care itself on gut physiology. It is a general truth that ICU patients have better outcomes in terms of wound healing, infectious complications, length of ICU stay and global costs when enteral feeding is successful (Lipman, 1998; Kreymann et al, 2006). For this to happen the gastrointestinal tract has to be functional, and any dysfunctions must be appropriately managed to allow enteral feeding to continue. This requires detailed insight into the underlying mediators of gut function.

The study of the gut has gradually embraced and incorporated fields such as endocrinology, neuro-immunology and molecular biology in an attempt to understand the intricate physiology and pathophysiology of the gastrointestinal tract. Thus, the presence of molecules (such as CRF) classically considered strictly as part of the central nervous system raises enticing questions about the mechanisms governing the effects of stressors on the digestive system. However, defining the specific roles of CRF synthesised or acting locally in the gut has actually been cumbered by the very fact that this molecule, once thought to be only a hypothalamic releasing factor, has been found throughout the body. Central or systemic application of CRF antagonists can impinge on all cell types that express CRF receptors, in all tissues. Reductionist research approaches and in vitro work on animals have already progressed the field. One further answer would be the generation of an animal model that lacks either the peptide or its receptor in particular cell sub-populations in the gastrointestinal tract. This is no simple task.

The very elegant work of La Fleur et al (2005) in using RNA interference *in vivo* was a step toward solving these difficulties, and similar work is yet to be done in man. Her work is an exciting observation of intestinal production of stress peptides usually associated only with the central nervous system. This data neatly reinforces the concept of the "enteric brain" – an idea which surpasses the notion simply of brain-gut connectedness, but effectively puts the brain at least partly - or perhaps functionally - within the gastrointestinal tract. The intestinal effects of local CRF demonstrated in La Fleur's work included motility effects and changes to the intestinal barrier state. It must be noted that evidence from human data for such an association, particularly in intestinal tissues, is to date speculative at best. This study has underlined the immense challenges involved in attempting to address these questions in human beings.

4.10. Concluding remarks

The gastrointestinal tract is no longer considered simply an access point for nutrients and a conduit for digestive waste products. Rather it is recognised to also be a system of organs of great immunological import, an efficient barrier of defence against pathogenic invasion of the internal environment, a regulator of fluid and electrolytes and a producer of hormones and other mediators with both local and systemic action. Breakdown in these integrated functions is a major problem in critically ill patients, and may even potentially threaten survival.

The impressive improvement in medical treatment provided to trauma patients as well as progressive measures in technological advancements in ICU care are creating a new form of sub-acute stress not previously seen in our hospitals, or experienced by human life – the extended critical illness (van den Berghe, 2002). Prolonged critical illness induces adaptations to HPA activity which impact upon neuroimmunoendocrine processes and metabolism. It is as yet unclear whether this is adaptive or maladaptive, and how this form of stress may impact on gut function in the ICU, either via centrally released CRF or via a peripherally mediated stress response system. Many questions remain. Yet, it is intriguing to consider that further interrogation of CRF activity may uncover mechanisms contributing to various gastrointestinal disorders.

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Appendix 1: Materials and Equipment

A1.1. Materials

12 x 75mm polypropylene tubes (Laboratory and Scientific Equipment Company, SA)
20G Introcan® intravenous disposable multiple sampling cannula (B.Braun, Germany)
20G Mandrin® Teflon disposable stylet (B.Braun, Germany)
40ml sterile disposable specimen container (Laboratory and Scientific Equipment Company, SA)
96 well sterile ELISA plate (Greiner Bio-one, Germany)
Cellstar® cryovials (Greiner Bio-one, Germany)
Disposable Pasteur Pipettes (Laboratory and Scientific Equipment Company, SA)
Disposable sterile 2ml syringe (Becton Dickinson, UK)
Disposable sterile 5ml syringe (Becton Dickinson, UK)
DNAse-, RNAse- free 0.6ml polypropylene microfuge tubes (Axygen Scientific, USA)
DNAse-, RNAse-free sterile polypropylene 1.5ml microcentrifuge tubes (Axygen Scientific, USA)
EDTA (lavender top) 5 ml Vacutainer® blood collection tubes (Becton Dickinson, UK)
Entellan® Rapid Embedding Agent (Electron Microscopy Sciences, USA)
Lactulose Syrup 3.3 g / 5 ml (Lacson®, Aspen Pharmacare, SA)
Mannitol 25% intravenous solution (Intramed Mannitol®, Bodene, SA)
Paracetamol tablets (Panado®, AI Self Med, SA)
Plain (red top) 5ml Vacutainer® blood collection tubes (Becton Dickinson, UK)
Sep-Pak® Plus C18 cartridges (Waters Corporation, USA)

A1.2. Equipment

ABI Prism™ 3100 Automated Sequencer (Applied Biosystems, USA)
Automated 24 hour tissue processor
DuraDry™ Freeze-dryer (FTS Systems, USA)
GeneAmps PCR System 2700 (Applied Biosystems, USA)
Hermle Z100M Pulser (Labnet International Inc, Korea)
Kodak DC 290 digital camera (Kodak, USA)
Multigene™ Gradient Thermal cycler (Labnet International Inc, Korea)
NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, USA)
Neslab Exacal water bath (Neslab Instruments Inc, USA)
PCR Sprint Thermal Cycler (Thermo Fisher Scientific, USA)
Polytron® (Kinematica, Switzerland)
Refridgerated Hermle Z233 MK-2 tabletop centrifuge (Labnet International Inc, Korea)
Refridgerated tabletop centrifuge Hermle Z233 MK-2 (Labnet International Inc, Korea)
Spectroline® Transilluminator TS-302 (Spectronics Corporation, USA)
Tabletop Heraeus Labofuge centrifuge (Heraeus Sepatech, Germany)
Tabletop microcentrifuge (Labnet International Inc, Korea)
Vortex Genie 2 (Scientific Industries Inc, USA))

Appendix 2: Reagents, Reactions and Recipes

A2.1. Reagents

AxSYM™ Acetominophen Assay Reagent Pack (Abbott Laboratories, USA)

BCA™ Protein Assay Kit (Pierce, USA)

Corticotropin-releasing factor (CRF) human RIA kit (Phoenix Pharmaceuticals Inc, USA)

GelPilot 50 bp Ladder or 100 bp Plus Ladder Molecular Weight Marker (Qiagen, USA)

DNA Molecular Weight Marker VIII (Roche Molecular Biochemicals, Germany)

SYBR® Gold Nucleic acid gel stain (Molecular Probes Inc, USA)

gDNA WipeOut Buffer (Qiagen, USA)

SuRE/Cut Buffer H (Roche Molecular Biochemicals, Germany)

TriPure™ Isolation Reagent (Boehringer Mannheim Corporation)

PstI Restriction Enzyme (Roche Molecular Biochemicals, Germany)

FailSafe™ Buffer (Epicentre Biotechnologies, USA)

Taq Polymerase (Bioline, USA)

Wizard SV Gel and PCR Clean Up System (Promega Corporation, USA)

Hi-Di™ Formamide (Applied Biosystems, USA)

A2.2. Reactions

Reverse Transcription (RT)

The following 1X reaction was set up on ice:

RNA mix: 1 µg RNA
1.5 µl gDNA WipeOut Buffer
Sterile water to total volume of 10.5 µl
1 µl oligo dT

RT mix (ImProm-II™ Reverse Transcription System kit, Promega Corporation, USA)
8.5 µl RT mix containing:
10 mM dNTP
25 mM $MgCl_2$
5X reaction buffer
20 U RNAse inhibitor
1 µl reverse transcriptase

Polymerase Chain Reaction (PCR)

The following 1X reaction was set up on ice:

0.5X FailSafe™ buffer
1 µl cDNA template
0.67 pmol forward primer (concentration of 2.5 pmol)
0.67 pmol reverse primer (concentration of 2.5 pmol)
0.5 U Taq polymerase
Sterile water to total volume of 7.5 µl

Restriction Enzyme Digestion

The following 1X reaction was set up:

10 µl purified PCR product
2 µl 10X SuRE/Cut Buffer H
6 µl sterile water
10 U PstI

Cycle Sequencing (BigDye® v3.1. Terminator Cycle Sequencing kit, Applied Biosystems, USA)

The following 1X reaction was set up on ice:

For sequencing, in a total volume of 20 µl:

2-5 µl purified PCR product
3 pmol forward primer
1X BigDye reaction buffer
1X termination mix

For analysis: 5 µl sequenced product
7 µl Hi-Di™ formamide

A2.3. Recipes

0.1M Acetic acid

5.72 ml glacial acetic acid
Make up to 1000ml with sterile deionised water

60% Acetonitrile in 1% TFA

600 ml acetonitrile
Make up to 1000 ml with 1% TFA

1% Acid alcohol

990 ml 70% alcohol
10ml concentrated hydrochloric acid

1% Agarose gel

1 g agarose powder (adjust for desired gel concentration)
90 ml 1x TBE or TAE buffer

Mix agarose with buffer and melt by heating in a microwave oven at 30 second intervals with gentle swirling until agarose is completely dissolved. Allow to cool and make volume up to final volume of 100 ml with buffer to correct for evaporation losses.

Bismarck Brown Solution

0.5 g Bismarck Brown, CI 21010
80 ml absolute alcohol
20 ml 1% hydrochloric acid

CRF PCR Primers (Whitehead Scientific (Pty) Ltd, South Africa)

100 µM stock (as supplied)

For 50 µM working stock dilute 1:2.

For PCR dilute 1:10 for 5µM primers and aliquot in 20 µl amounts. Store at -20°C.

Diethylpyrocarbonate (DEPC)-treated distilled water

100 µl DEPC

1000 ml distilled water

Mix DEPC with distilled water. Autoclave to sterilise and inactivate DEPC.

Eosin/Phloxine solution

Solution A: 1% Eosin

10 g Eosin Y, Cl 45380

1000 ml distilled water

Solution B: 1% Phloxine

1 g Phloxine B, Cl 45410

100 ml distilled water

Mix 100 ml of Solution A plus 10 ml of Solution B. Allow mixture to ripen for no less than 2 weeks before using. Dilute this mixture 1:1 with distilled water and allow to stand for a further two weeks to prevent fading. The stain is now ready for use.

Mayer's Haemalum

1 g haematoxylin

0.2 g sodium iodate

50 g anhydrous potassium alum

50 g chloral hydrate

2 g citric acid

1000 ml distilled water

Dissolve the haematoxylin, potassium alum and sodium iodate in distilled water with gentle heat and stirring, of by allowing to stand overnight at room temperature. Add chloral hydrate and citric acid, boil for 5 minutes. Cool. Filter. Store in a dark cupboard.

4% Paraformaldehyde (PFA)

4 g paraformaldehyde (PFA)

100 ml PBS

Weigh out PFA wearing gloves and mask in a fume hood. Make up volume to 100ml with 1X PBS. Place in a beaker and heat at 50°C while stirring on a magnetic stirrer until solution is clear. Store at 4°C.

1X Phosphate Buffered Saline (PBS)

8 g NaCl

1.26 g anhydrous Na_2HPO_4

0.2 g KH_2PO_4

Make up to 1000 ml with distilled water. Adjust pH to 7.4.

QuantiTect® Primer Assays for GAPDH and GUS (Qiagen, USA)

To reconstitute the 10X (as supplied) primers, add 1.1 ml TE pH 8.0 and vortex.

Aliquot and store at -20°C until use.

5X Sample Loading Dye (agarose gels)

5.74ml glycerol

1 ml 10X TBE

0.1 ml 0.25% bromophenol blue

Make up to 10 ml with deionised water. Filter through sterile 0.45µm filter and store at -20°C in 1ml aliquots.

Scott's Tap Water Substitute

3.5 g sodium bicarbonate

20 g magnesium sulphate

1000 ml distilled water

Pinch of thymol

Dissolve the sodium bicarbonate and magnesium sulphate in the distilled water. Preserve with a pinch of thymol.

Trasylol working solution

1 ml Trasylol®, Bayer (Pty) Ltd, Germany

9ml sterile 0.9% NaCl

This is a working solution of 38.5 TIU/ml.

1% Trifluoroacetic acid (TFA)

20ml TFA added to 100 ml sterile deionized water in a fume hood.

Make up final volume to 2000 ml with sterile deionised water

10X Tris/Acetate/EDTA (TAE) Buffer

48.9 g Tris base

10.9 glacial acetic acid

2.92 g EDTA, pH 8

1000 ml distilled water

Autoclave to sterilise. Dilute 1:10 for 1X buffer.

10X Tris/Borate/EDTA (TBE) Buffer

54 g Tris base

27.5 g Boric Acid

2.92 g EDTA, pH 8

1000 ml distilled water

Autoclave to sterilise. Dilute 1:10 in double-distilled water for 1X buffer.

Appendix 3: Trauma Scoring Systems

A3.1. APACHE II Score

See Table A1. below for the APACHE II scoring system (Knaus et al, 1985).

Table A1. APACHE II SCORE

Score worst values for 24 hours following ICU admission

PHYSIOLOGICAL VARIABLE	HIGH ABNORMAL RANGE					LOW ABNORMAL RANGE			
	+4	+3	+2	+1	0	+1	+2	+3	+4
Temperature – rectal (°C)	≥ 41	39-40.9		38.5-38.9	36-38.4	34-35.9	32-33.9	30-31.9	≤ 29.9
Mean Arterial Pressure – mmHg	≥ 160	130-159	110-129		70-109		50-69		≤ 49
Heart rate	≥ 180	140-179	110-139		70-109		55-69	40-54	≤ 39
Respiratory rate(ventilated or non-ventilated)	≥ 50	35-49		25-34	12-24	10-11	6-9		≤ 5
Oxygenation: A-aDO_2 pr PaO_2 (mmHg) a. FiO_2 ≥ 0.5 record A-aDO_2	≥ 66	46-65	26-46		<26				
b. FiO_2 ≤ 0.5 record only PaO_2					>9.3	8.1-9.3		7.3-8.0	< 7.3
Arterial pH	≥ 7.7	7.5-7.69		7.5-7.59	7.33-7.49		7.25-7.32	7.15-7.24	< 7.15
Serum sodium (mmol/l)	≥ 180	160-179	155-159	150-154	130-149		120-129	111-119	< 110
Serum potassium (mmol/l)	≥ 7	6-6.9		5.5-5.9	3.5-5.4	3-3.4	2.5-2.9		< 2.5
Serum creatinine (mg/100ml) (double point score for acute renal failure)	≥ 309	177-308	133-176		53-132		<53		
Hematocrit (%)	≥ 60		50-59.9	46-49.9	30-45.9		20-29.9		< 20
White blood count (total in 1000s/mm^3)	≥ 40		20-39.9	15-19.9	3-14.9		1-2.9		< 1
Serum HCO_3 (venous mmol/l) (not preferred, use only if no blood gas)	≥ 52	41-51.9		32-40.9	22-31.9		18-21.9	15-17.9	< 15

SCORING	POINTS
Acute Physiology score (APS) – sum of 12 individual points above	
Glasgow Coma Score (GCS): Score = 15-GCS	
Age points: assign as follows: ≤ 44 yrs = 0; 45-54 = 2; 55-64 =3; 65-74 = 5; ≥ 75 = 6	
Chronic Health Points: assign as follows: - If the patient has a history of severe organ system insufficiency or is immuno-compromised assign 5 points for non-operative or emergency post-operative patients OR 2 points for elective post-operative patients. Organ insufficiency must have been evident prior to this hospital admission and conform to the criteria below.	
LIVER: biopsy proven cirrhosis and documented portal hypertension; episodes of past upper GI bleeding attributed to portal hypertension; or prior episodes of hepatic failure/encephalopathy/coma	
CARDIOVASCULAR: New York Heart Association Class IV / RENAL: receiving chronic dialysis	
RESPIRATORY: Chronic restrictive, obstructive or vascular disease resulting in severe exercise restriction i.e. unable to climb stairs or perform household duties; or documented chronic hypoxia, hypercapnia; secondary polycythemia, severe pulmonary hypertension > 40 mmHg or respiratory dependency	
IMMUNO-COMPROMISED: The patient has received therapy that suppressed resistance to infection e.g.immuno-suppression, chemotherapy, radiation, long term or high dose steroids, or has a disease that is sufficiently advanced to suppress resistance to infection e.g. leukaemia, lymphoma, AIDS	
TOTAL	

A3.2. Injury Severity Score

The Injury Severity Score (ISS) is a system for scoring multiple injuries according to the severity of injury in each of six body regions. In each anatomical region, injuries are scored according to the Abbreviated Injury Scale (AIS) developed by the American Medical Association Committee on Medical Aspects of Automotive Safety on a scale of 1-6 as indicated in Table A2 and Table A3 below.

Table A2. AIS scoring categories

AIS	Injury
1	Minor
2	Moderate
3	Severe: not life-threatening
4	Severe: life-threatening
5	Critical: survival uncertain
6	Unsurvivable

The ISS is then calculated by selecting the highest injury score for each of the six body regions (see Table A3 for detail). Of those, the overall highest three scores are selected. These three scores are each scored and then added together to provide the final ISS as indicated by the following calculation:

$$ISS = (\text{highest region score})^2 + (\text{second highest region score})^2 + (\text{third highest region score})^2$$

The minimum score is zero and maximum possible score is 75. If any injury in any body region is assigned an AIS of 6, the ISS is automatically scored 75 (Baker et al, 1974).

Table A3 below lists examples of specific injuries scoring AIS 1-6 in each of the anatomical regions.

Table A3. AIS Scores according to injury and body region

Blunt Injury					
Score	1 Minor	2 Moderate	3 Severe: not life-threatening	4 Severe: life-threatening	5 Critical: survival uncertain
Head/neck	Headache/dizziness 2° to head trauma Cervical spine strain with no fracture or dislocation	Amnesia from accident Lethargic/stuporous/obtunded but can be roused by verbal stimuli Unconscious < 1hr Simple vault fracture Thyroid contusion Brachial plexus injury Dislocation or fracture spinous or transverse process of C-spine Minor compression fracture (<20%) C-spine	Unconscious 1-6 hrs Unconscious < 1hr with neurological deficit Fracture base of skull Comminuted, compound or depressed vault fracture Cerebral contusion/subarachnoid haemorrhage Intimal tear/thrombosis carotid artery Contusion larynx/pharynx Cervical cord contusion Dislocation or fracture of lamina body, pedicle or facet of C-spine Compression fracture > 1 vertebra or >20% anterior height	Unconscious 1-6 hrs with neurological deficit Unconscious 6-24 hrs Appropriate response only to painful stimuli Fractured skull with depression >2cm, torn dura or tissue loss Intracranial haemotoma <100ml Incomplete cervical cord lesion Laryngeal crush Intimal tear/thrombosis carotid artery with neurological deficit	Unconscious with inappropriate movement Unconscious > 24 hrs Brain stem injury Intracranial haematoma >100ml Complete cervical cord lesion C4 or below
Face	Corneal abrasions Superficial tongue laceration Nasal or mandiular ramus fracture* Tooth fracture/avulsion or dislocation	Zygoma, orbit*, body* or subcondylar mandible fracture LeFort I fracture Scleral/corneal laceration	Optic nerve laceration LeFort II fracture	LeFort III fracture	
Thorax	Rib fracture* Thoracic spine strain Rib cage contusion Sternal contusion	2-3 rib fractures* Sternum fracture Dislocation or fracture spinous or tranverse process T-spine Minor compression fracture(<20%) T-spine	Lung contusion/laceration <1 lobe Unilateral haemo-/pneumothorax Diaphragm rupture 4 rib fractures* Intimal tear/minor laceration/thrombosis subclavian or innominate artery Inhalation burn, minor Dislocation or fracture of lamina body, pedicle or facet of T-spine Compression fracture > 1 vertebra or >20% anterior height Cord contusion with transient neurological signs	Multilobar lung contusion or laceration Haemo-/pneumomediastinum Bilateral haemo-pneumothorax Flail chest Myocardial contusion Tension pneumothorax Haemothorax >1000ml Tracheal fracture Intimal aortic tear Major laceration subclavian or innominate artery Incomplete cord syndrome	Major aortic laceration Cardiac laceration Rupture bronchus/trachea Flail chest/inhalation burn requiring mechanical support Laryngotracheal separation Multilobar lung laceration with tension pneumothorax, haemo-/pneumomediastinum or > 1000ml haemothorax Cord laceration or complete cord lesion
	* add AIS 1 if associated with haemothorax, pneumothorax or haemo-/pneumo-mediastinum				
Abdomen	Abrasion/contusion/superficial laceration scrotum, vagina, vulva, perineum Lumbar spine strain Haematuria	Contusion/superficial laceration stomach/ mesentery/ small bowel/ bladder/ ureter/ urethra Minor contusion/laceration kidney/ liver/ spleen/pancreas Contusion duodenum/colon Dislocation or fracture spinous or transverse process L-spine Minor compression fracture(<20%) L-spine Nerve root injury	Superficial laceration duodenum/colon/rectum Perforation small bowel/mesentery/bladder/ureter/urethra Major contusion/minor laceration with major vessel involvement or haemo-peritoneum >1000ml of kidney/liver/spleen/pancreas Minor iliac artery/vein laceration Retroperitoneal haemotoma Dislocation or fracture of lamina body, pedicle or facet of L-spine Compression fracture > 1 vertebra or >20% anterior height Cord contusion with transient neurological signs	Perforation stomach/duodenum/colon/rectum Perforation with tissue loss stomach/ bladder/small bowel/ureter/urethra Major liver laceration Major iliac artery or vein laceration Incomplete cord syndrome Placental abruption	Major laceration with tissue loss or gross contamination of duodenum/colon/rectum Complex rupture liver/spleen/kidney/pancreas Complete cord lesion
Extremities	Contusion elbow/shoulder/wrist/ankle Fracture/dislocation finger/toe Sprain A-C joint/shoulder/elbow/ finger/wrist/hip/ankle/toe	Fracture humerus*/ radius*/ ulna*/ fibula/tibia*/ clavicle/ scapula/ carpals/ metacarpals/ calcaneous/ tarsals/ metatarsals/ pubic rami/ simple pelvic fracture Dislocation elbow/hand/shoulder/A-C joint Major muscle/tendon laceration Intimal tear/minor laceration axillary/brachial/popliteal artery/axillary/femoral/popliteal vein * Add AIS 1 if open/displaced/comminuted	Comminuted pelvic fracture Fractured femur Dislocation wrist/ankle/knee/hip Below knee or upper extremity amputation Rupture knee ligaments Sciatic nerve laceration Intimal tear/minor laceration femoral artery Major laceration and/or thrombosis axillary or popliteal artery, popliteal or femoral vein	Pelvic crush fracture Traumatic above knee amputation/crush injury Major laceration femoral or brachial artery	Open pelvic crush fracture
External	Abrasions/contusions <25cm on face/hand or <50cm on body Superficial lacerations <5cm on face/hand or <10cm on body 1° burn up to 100% 2° or 3° burn/degloving injury 10% total body	Abrasions/contusions >25com face/hand or >50cm on body Laceration >5cm on face/hand or >10cm on body 2° or 3° burn/degloving injury 10-19% total body	2° or 3° burn/degloving injury 20-29% total body	2° or 3° burn/degloving injury 30-39% total body	2° or 3° burn/degloving injury 40-89% total body

AIS = 6 MAXIMUM INJURY AUTOMATICALLY ASSIGNED ISS = 75

Head/Neck: Crush fracture, crush/laceration brain stem. Decapitation. Cold crush/laceration or total transection with/without fracture C3 or above.

Thorax: Total severance aorta. Chest massively crushed

Abdomen: Torso transection

External: 2° or 3° burn/degloving injury >90% total body surface

ISS calculation

Body region	AIS	AIS²
Head/neck	.....	.....
Face	.....	.....
Thorax	.....	.....
Abdomen	.....	.....
Extremities	.....	.....
External	.....	.....

ISS (sum of squares of 3 worst scores) =

Penetrating Injury					
Score	1 Minor	2 Moderate	3 Severe: not life-threatening	4 Severe: life-threatening	5 Critical: survival uncertain
Head/neck		Penetrating injury to neck with no organ involvement	Complex penetrating injury to neck with tissue loss/organ involvement Minor laceration carotid/vertebral artery/internal jugular vein Transection and/or segmental loss jugular vein Thyroid laceration Superficial laceration larynx/pharynx Cord contusion with transient neurological signs	Minor laceration carotid/vertebral artery with neurological deficit Transection carotid/vertebral artery/internal jugular vein Segmental loss internal jugular vein Perforation larynx/pharynx Cord contusion with incomplete cord syndrome	Penetrating injury with entrance and exit wounds Penetrating injury of cerebrum/cerebellum Segmental loss carotid/vertebral artery Complex laceration larynx/pharynx Cord laceration Complete cord lesion
Face	Penetrating injury with no tissue loss	Penetrating injury with superficial tissue loss Corneal/scleral laceration	Penetrating injury with major tissue loss		
Thorax	Penetrating injury with no violation of pleural cavity	Thoracic duct laceration Pleural laceration	Complex penetrating injury but no violation of the pleural cavity Superficial laceration innominate/pulmonary/subclavian and other named smaller vessels Superficial laceration trachea/bronchus/oesophagus Lung laceration <1 lobe Unilateral haemo or pneumothorax Diaphragmatic laceration Cord contusion with transient neurological signs	Superficial aortic laceration Major laceration innominate/pulmonary/subclavian and other named smaller arteries/vena cava/brachiocephalic/pulmonary/ subclavian and other smaller veins Transection/tissue loss and other named smaller veins Perforation trachea/bronchus/oesophagus Multilobar lung laceration Haemo-/pneumomediastinum Bilateral haemo-& pneumothorax Tension pneumothorax Haemothorax >1000ml Cardiac tamponade Cord contusion with incomplete cord syndrome	Major aortic laceration Transection/segmental loss vena cava/pulmonary/ brachiocephalic vein and other smaller named arteries Laceration trachea/bronchus/oesophagus with tissue loss Multilobar lung laceration with tension pneumothorax >1000ml Myocardium/valve laceration Cord laceration Complete cord lesion
Abdomen	Penetrating injury with no peritoneal penetration	Penetrating injury with superficial tissue loss but no peritoneal penetration Superficial laceration stomach/small bowel/bladder/mesentery/liver/ureter/kidney/ spleen/pancreas Laceration through peritoneum	Penetrating injury with significant tissue loss but no peritoneal penetration Superficial laceration vena cava/iliac and other named smaller arteries and veins Superficial laceration duodenum/colon/rectum Full thickness laceration small bowel/mesentery/bladder/ureter Major/minor laceration with major vessel injury >1000ml haemoperitoneum kidney/liver/spleen/pancreas Cord contusion with transient neurological signs	Minor aortic laceration Major laceration vena cava/iliac and other named smaller arteries and veins Transection/segmental loss iliac and other named smaller veins Full thickness laceration stomach/colon/duodenum/rectum Tissue loss/gross contamination stomach/small bowel/mesentery/bladder/ureter Cord contusion with incomplete cord syndrome	Major aortic laceration Transection/segmental loss vena cava/iliac and other named smaller arteries Tissue loss/gross contamination duodenum/colon/rectum Tissue loss kidney/live/spleen/pancreas Cord laceration
Extremities	Superficial laceration brachial or other named veins	Simple penetrating injury with no internal structure involvement Superficial laceration axillary/popliteal artery or axillary/femoral/popliteal vein Major laceration and/or segmental loss brachial vein and other named smaller vein and arteries Laceration median/radial/ulnar/femoral/tibial/peroneal nerves Major tendon/muscle laceration	Complex penetrating injury with internal structure involvement Superficial laceration femoral artery Major laceration axillary/popliteal artery or axillary/femoral/popliteal vein Sciatic nerve laceration >1 nerve laceration in same extremity Multiple tendon/muscle lacerations in same extremity	Major laceration femoral or brachial artery Segmental loss brachial/axillary/popliteal artery	Segmental loss femoral artery
External	Superficial laceration <5cm on face/hand or <10cm on body Penetrating injury with no tissue loss	Laceration >5cm on face/hand or >10cm on body Penetrating injury with superficial tissue loss			

AIS = 6 MAXIMUM INJURY AUTOMATICALLY ASSIGNED ISS = 75

Head/Neck: Brainstem laceration

Thorax: Aortic transection. Segmental loss aortic/innominate/pulmonary/subclavian arteries

Abdomen: Aortic transection

ISS calculation

Body region	AIS	AIS^2
Head/neck	.....	.....
Face	.....	.....
Thorax	.....	.....
Abdomen	.....	.....
Extremities	.....	.....
External	.....	.....

ISS (sum of squares of 3 worst scores) =

(Civil and Schwab, 1988)