THE EFFECT OF *HELICOBACTER PYLORI* ON ENDOTHELIAL CELL PROPERTIES AND FUNCTIONS: A PUTATIVE ROLE IN VASCULAR DYSFUNCTION.

A dissertation submitted for the degree of Ph.D by

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Chapter 1

Introduction

1.0 Cardiovascular Disease

Cardiovascular disease (CVD) refers to any abnormal condition characterized by the dysfunction of the heart or blood vessels. It includes diseases such as atherosclerosis, coronary heart disease, hypertension and cerebrovascular disease. It is the number one cause of death in the United States and most European countries, with World Health Organisation statistics estimating 16.7 million people per annum around the globe die from the disease. This is 29% of all deaths worldwide per year. A study by Leal *et al.* (Leal et al., 2006) revealed that cardiovascular disease costs the economy of the European Union €169 billion per year; this includes healthcare costs, lost productivity and informal healthcare costs. Figures released by the Irish Heart Foundation covering a 50-year period have provided evidence of prolonged high mortality rates due to this disease, however it also revealed that progress has been made and rates continue to decrease.

In 2006, 9,662 people died from cardiovascular diseases in Ireland; figures from 1950 show 11,887 deaths from the same group of diseases. While today's numbers remain high, they are in fact declining at a steady rate. For example, mortality rates from ischemic heart disease (IHD) reached a peak in the mid nineteen-seventies, which continued until 1985, but has been decreasing ever since. In fact, approximately 37% fewer men and 30% fewer women are now dying from IHD (IrishHeartFoundation, 2001.).

The improving figures are due not only to advances in treatment and preventative strategies, but also to greater public health awareness. The risk factors associated with the development and initiation of cardiovascular disease are well

known and heavily advertised. Interestingly, population based studies have identified lesions appearing in the right coronary arteries of youths in the 15-19 age bracket (Vanhecke *et al*., 2006). If mortality rates are to decrease significantly, more emphasis needs to be placed on targeting this group with information on preventative measures and lifestyle choices, which can significantly lessen their chances of cardiovascular disease.

Risk factors for the disease include: age, gender, smoking, elevated cholesterol levels, obesity, high blood pressure, stress, depression and an absence of key nutritional elements. Numerous measures can be taken to protect against these factors, all of which correspond to a healthier lifestyle: cessation of smoking, regular exercise and healthy diet with inclusion of oily fish (Reiner et al., 2007) and olive oil.

Much study has focused on the manner of initiation and progression of cardiovascular disease, from the effects of blood-flow-associated hemodynamic forces (Cunningham and Gotlieb, 2005), cholesterol, and the immune system (Cullen *et al*., 2005, Pasqui *et al*., 2005) to the role of pathogens (Ando *et al*., 2006) and cell function (Cummins *et al*., 2007). Central to all of this research are the cells that line the vasculature which sense and respond to circulatory conditions (both humoral and mechanical)- namely, the endothelial cell monolayer or endothelium.

1.1 The Endothelium

The vascular endothelial monolayer or endothelium is a dynamic cellular interface between the vessel wall and bloodstream. In addition to regulating the physiological effects of humoral and mechanical stimuli on vessel tone and remodeling (Alexander and Elrod, 2002), the endothelium participates in immune and inflammatory reactions, presents a non-thrombogenic surface for blood flow, and constitutes a highly effective fluid and solute barrier (Sagripanti and Carpi, 2000, Traub and Berk, 1998, van Hinsbergh, 2001).

The basic structure of the endothelial cell is similar to that of other human cell types. The passage of molecules between the blood and sub-endothelial space is regulated by intercellular tight junctions and adhesion junctions. Actin stress fibers that cross the cytoplasm act to regulate cell morphology in response to blood flow-associated hemodynamic forces (e.g. in situations of increased flow, and hence shear stress, the cells will be flattened and aligned in the direction of flow). The endothelium interacts not only with the extracellular matrix (the extracellular part of animal tissue that provides structural support for cells and contains proteoglycans, collagens, laminins, fibronectin and elastin) but has been shown to indirectly interact with other vascular cell types such as smooth muscle cells (SMC) (Cummins *et al*., 2007), influencing their behaviour.

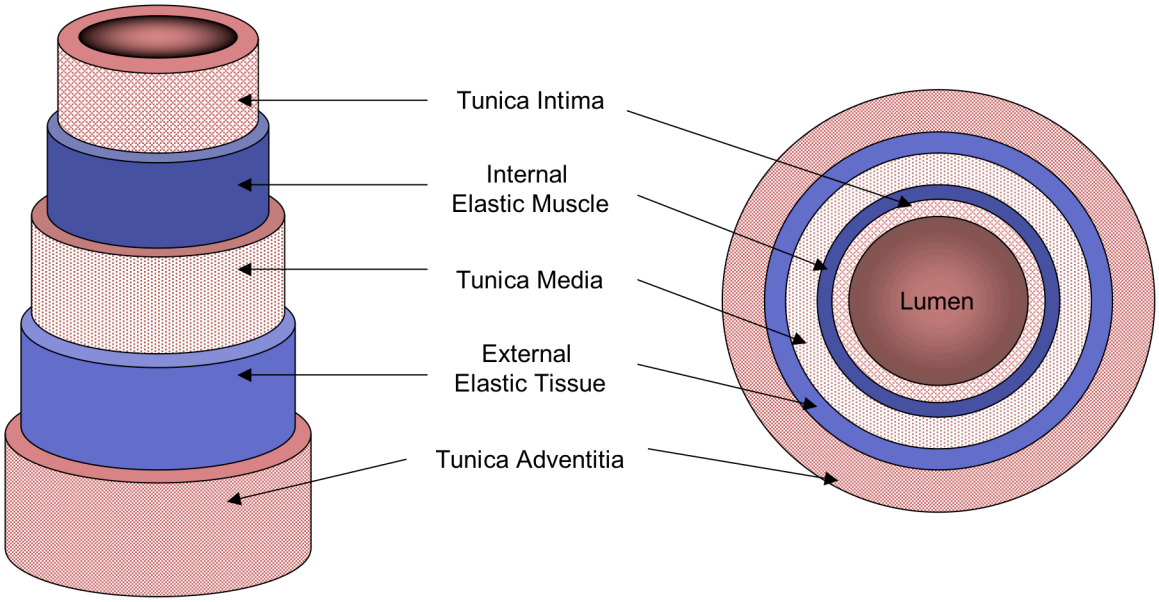


Fig. 1.1.1 The Human Blood Vessel. Blood vessels function as part of the circulatory system to transport blood throughout the body. They are comprised of three main layers **Tunica intima:** This is the innermost layer and consists of a single layer of endothelial cells and a sub-endothelial connective tissue containing scattered SMCs. It is separated from the next layer by an **internal elastic lamina**. **Tunica media:** This layer is comprised of helically arranged SMCs and elastic collagen fibers. The **external elastic lamina** separates it from the next layer. **Tunica Adventita:** The outermost layer of a blood vessel. It contains fibroblasts and elastic collagen fibers.

The human blood vessel is shown in Fig. 1.1.1, and consists of three main layers- the tunica intima, tunica media and tunica adventita. The most important function of the endothelial cells lining the tunica intima is modulation of vessel tone and maintenance of homeostasis. It does so by producing vasodilators, vasoconstrictors, pro- and anti-coagulants, pro- and anti-inflammatory molecules, reactive oxygen species and anti-oxidants. When endothelial cells lose the ability to control

homeostasis, it is indicative of endothelial dysfunction and initiation of a pathologic condition may occur (Esper *et al*., 2006).

1.2 Endothelial Dysfunction

Vasodilation and vasoconstriction represent the main method of endothelial vessel tone control. Central to these mechanisms is the action of nitric oxide (NO), a volatile, biologically active gas with a half-life of seconds, which is present in virtually all tissue. Importantly, attenuation of NO is one of the earliest biochemical changes preceding endothelial dysfunction (Taddei *et al*., 2006). Three nitric oxide synthase (NOS) isoforms have been identified: neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed, while inducible NOS (iNOS) is regulated by cytokine stimulation. NO is produced by the action of NO which catalyse the NADPH-dependent conversion of L-arginine and O_2 to L-citrulline and NO. NO then crosses the endothelial intima to the SMC layer. Here, through nitrosilation of the heme moiety from guanylate-cyclase, which subsequently converts GTP to cGMP, regulation of cytosolic Ca^{2+} (by cGMP) is achieved and smooth muscle fibre relaxation and vasodilation occur (Loscalzo and Welch, 1995). The major stimulation for NO release arises from hemodynamic forces within the vessel.

The normal functions of endothelial cells include mediation of coagulation (Strukova, 2006), platelet adhesion (Celi et al., 1997), immune function (Stoll and Bendszus, 2006), and control of volume and electrolyte content of the intra and extravascular spaces (Eggermont et al., 2001), in addition to vaso-control of the

blood vessel (Lundberg and Weitzberg, 2005). Moreover, maintenance of vascular endothelial cell migration, proliferation, angiogenesis and apoptosis in the blood vessel is essential to vessel health and prevention of disease. Undisturbed laminar blood flow produces a shearing effect or "frictional drag" due to its lateral momentum on the endothelium; in this manner, it stimulates production of NO and is thus "atheroprotective". This ability of the endothelium to sense and respond to shear is mediated through mechanotransduction signalling mechanism, which include integrins, ion channels, G-proteins and Receptor Tyrosine Kinases. However, in regions of the vasculature such as arterial bifurcations or vessel curvatures, blood flow (and thus, shear) is turbulent and vessel homeostasis becomes imbalanced. In these areas, NO production is lowered, resulting in weakened cell-cell contacts, dysregulation of endothelial growth and functional parameters, platelet adhesion, neutrophil infiltration and LDL influx. Thus, locations where perturbed shear predominates are implicated in initiation of endothelial dysfunctional cardiovascular disease states (e.g. atherosclerosis).

In addition to shear, other risk factors are also implicated in plaque initiation and progression. The role of infectious agents for example and in particular the bacterium *Helicobacter pylori* has been proposed as a risk factor for atherosclerotic lesion formation, by possibly inducing and/or exacerbating endothelial dysfunction.

In the following section we will examine the evidence linking shear stress, mechanotransduction and *H. pylori,* to a pro-atherosclerotic cell phenotype. Moreover we examine their ability to act in a synergistic fashion, resulting in endothelial dysfunction and a disease state.

1.2.1 Hemodynamics

The main components of the circulatory system are the heart, blood and blood vessels. Its function is transport of oxygenated blood to all major organs of the body, and transport of deoxygenated blood from these organs back to the heart for reoxygenation. Blood flowing in this system exerts two major forces on blood vessels, namely cyclic strain and shear stress.

Cyclic strain is the circumferential stretch, which is exerted tangentially to the direction of flow on the vessel wall and is caused by the pulsatile nature of blood flow from the heart. It is directly related to blood pressure and vessel dimensions and can be described by Laplace's Law (Laplace, 1899):

$$T = \frac{\mathrm{Pr}}{h}$$

Where T is the wall tension (or force per unit length of the vessel), P is the blood pressure, r is the radius and h is the thickness of the vessel wall. Unlike shear stress which impacts exclusively on the endothelium, cyclic strain imparts on both the endothelium and smooth muscle layers simultaneously. Endothelium metabolism is profoundly affected by cyclic strain, inducing alterations in gene expression that lead to changes in cell properties, function and vessel wall homeostasis (Chien *et al.*, 1998, Patrick and McIntire, 1995). Specifically, cyclic strain regulates the production a number of factors which directly impact on the endothelium; NO, NOS and cyclooxygenase 2 (Cox-2) (Cheng *et al.*, 2001, Coen *et al.*, 2004, Kito *et al.*, 1998). These act in modulating vessel diameter; platelet-derived growth factor

(PDGF) and vascular endothelial growth factor (VEGF) (Sumpio *et al*., 1998, Zheng *et al*., 2001)- are central to endothelial proliferation; RGD-dependent integrins, monocyte chemotactic protein (MCP)-1 and matrix metalloproteinases (Ulfhammer *et al*., 2005, von Offenberg Sweeney *et al*., 2004, Wung *et al*., 1997)- involved in cell migration and angiogenesis; intercellular adhesion molecule (ICAM)-1, zonula occludens (ZO)-1, and occludin (Collins *et al*., 2006, Pradhan and Sumpio, 2004)- central for cell-cell communication and barrier function. Importantly, physiological levels of cyclic strain have been shown to increase both migration (Von Offenberg Sweeney *et al*., 2005) and proliferation (Iba *et al*., 1991), whilst also decreasing apoptosis in aortic endothelial cells (Haga *et al*., 2003).

Blood flow exerts a frictional force on the luminal surface of the endothelium. This frictional drag is referred to as shear stress and is defined in terms of blood viscosity and velocity and is computationally estimated in units of dyne/cm^2. In the case laminar flow shear stress is expressed as:

$$\tau = \frac{4\mu Q}{\pi r^3}$$

Where μ is the viscosity, Q the flow rate and r the vessel radius (Lehoux and Tedgui, 2003). Under normal physiological conditions the mean shear stress to which the endothelium is exposed remains a relatively constant value of 10-15 dyne/cm^2. Under these conditions, endothelial cell stability and survival is promoted, the cells align in the direction of flow, and production of anti-thrombotic and vasodilatory

factors occur. These factors include prostacyclin, NO, and tissue plasminogen activator (tPA). Stimulation leading to production of NO is relatively simple- laminar flow opens specialised ion channels within the endothelial cell membrane such as Ca^{2+}- activated K^{+} channels. This hyperpolarises the cell, increasing the driving force for Ca^{2+} entry and activating the NOS III (eNOS) and hence NO production.

Perturbed or high levels of shear stress (due to turbulent or oscillatory flow), in conjunction with known risk factors for cardiovascular disease, can trigger signal transduction events leading to endothelial dysfunction and initiation of a pathological condition such as atherosclerosis. This type of shear (or altered hemodynamic) occurs at curvatures in the blood vessel and in arterial branch ostia and bifurcations (Davies *et al.*, 1995, Lehoux and Tedgui, 2003, Traub *et al.*, 1999) and results in attenuation of NO production along with increased reactive oxygen species (ROS) production.

ROS includes oxygen ions, free radicals and peroxides and are highly reactive molecules known to play important roles in cell signalling. Under laminar flow conditions ROS production is balanced by cellular antioxidant generation, preventing oxidant damage to the cell. However a shift in endothelial homeostasis due to aberrant shear stress upsets the balance leading to an excess of ROS and an oxidative stress state. LDL-cholesterol is easily oxidized when a state of oxidative stress exists, giving rise to LDL-ox, a highly immunogenic molecule found in all atherosclerotic lesions (Janeway and Medzhitov, 2002, Peiser *et al.*, 2002). Moreover LDL-ox is known to increase production of endothelium molecules and

monocyte chemoattractants (Li and Mehta, 2000), which have a cytotoxic effect on the endothelium, increase proinflammatory gene activation, and promote endothelial dysfunction, platelet aggregation, MMP expression and thrombogenesis (Tsimikas and Witztum, 2001). In short, excessive ROS production results in an inflammatory pro-atherogenic cell phenotype.

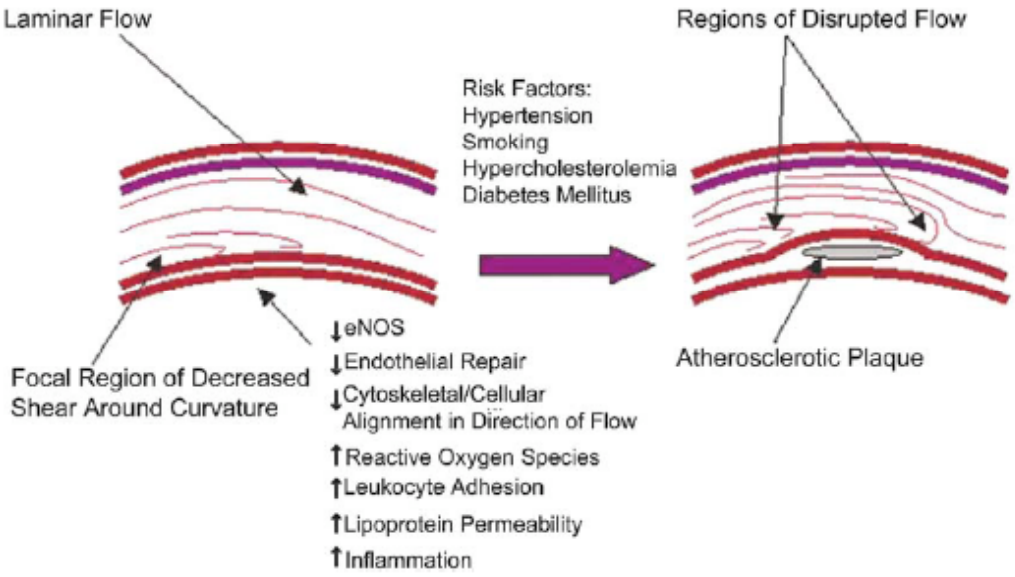


Fig. 1.2.1 The effect of laminar flow on the endothelium (Cunningham and Gotlieb, 2005).

1.2.1.1 Mechanotransduction

The manner in which a cell converts a mechanical signal such as shear stress into biochemical activity and subsequent cellular responses is known as mechanotransduction (Katsumi *et al*., 2004, Liu *et al*., 1996) and many studies have focused on the force-dependent signalling mechanisms involved. Current knowledge on the endothelial mechanosensors involved and their downstream effects is outlined overleaf.

1.2.1.2 Integrins

Integrins are membrane proteins, and comprise a family of over twenty transmembrane heterodimers (composed of two distinct chains- denoted alpha (α) and beta (β) subunits). They function in attachment of the endothelial cell to both the extracellular matrix (ECM) and other cells, in addition to their role in signal transduction from the ECM to the cell. The integrin extracellular domain binds to ECM ligands such as fibronectin, vitronectin and collagen, whilst the cytoplasmic domain interacts with signalling molecules in focal adhesion sites such as focal adhesion kinase (FAK) and c-Src (Richardson and Parsons, 1996). These distinctive features mean the exterior and interior of the cell are physically linked, allowing for bi-directional transmission of mechanical and biochemical signals across the plasma membrane. The binding of ECM ligands to integrin receptors results in an activation of signalling cascades resulting in cooperative regulation of cell functions such as adhesion, migration, growth and differentiation.

Cyclic strain of SMCs grown on vitronectin or fibronectin induces integrin-dependent proliferation, which can be inhibited with anti-β5 or anti-αvβ3 antibodies. However, SMCs grown on elastin or laminin do not proliferate under the same conditions (Wilson *et al*., 1995). Conversely, cyclic strain of SMCs on elastin caused a down-regulation in expression of c-*fos* and decreased cell proliferation (Reusch *et al*., 1996). Thus, responses to mechanical force have been shown to be dependent on specific integrin-ECM interactions (von Offenberg Sweeney *et al*., 2004).

In endothelial cells shear stress activates endothelial integrins as indicated by their clustering (Wang *et al*., 2002), association with adapter protein Shc (Chen *et*

al., 1999) and binding with WOW-1, an antibody specific to activated αvβ3 (Tzima *et al*., 2001). In addition, shear-induced activation of NF-κB is inhibited by anti-αvβ3 antibodies (Bhullar *et al*., 1998). Importantly, blocking integrins using anti-integrin antibodies or inhibitory RGD peptides, abrogates shear-induced signalling and its subsequent role in cellular function (Bhullar *et al*., 1998, Girard and Nerem, 1995, Liu *et al*., 2002).

1.2.1.3 Ion Channels

Ion channels are membrane protein complexes that enable transport of ions across biological membranes and in doing so, establish and control small voltage gradients. In this context, they are also involved in cardiac, skeletal and smooth muscle contraction, and epithelial nutrient and ion transport. Two different channels have been identified in endothelial cells that are responsive to hemodynamic forces- shear activated potassium channels and cyclic strain-activated channels (Sackin, 1995).

Birukov *et al*. (Birukov et al., 1995) revealed a role for ion channels in SMC mechanotransduction through the use of gadolinium- a specific blocker of the stretch-activated (SA) ion channels, which reduced expression of cyclic strain-induced SMC marker protein.

The effect of shear stress on endothelial cells, as noted previously, is to open inwardly rectifying K^+ channels and outwardly rectifying Cl^- ion channels (Barakat *et al*., 2006), thereby increasing Ca^{2+} influx into endothelial cells (Helmlinger *et al*., 1996, Kanai *et al*., 1995, Yamamoto *et al*., 2000), with consequences for cell signalling and functions. However, the precise mode of action involved in the

opening and closing of these channels remains unknown. Complicating this is the manner in which shear and cyclic strain can act synergistically or antagonistically, through differential activation of ion channels, under both laminar and perturbed flow.

1.2.1.4 G-proteins

G-proteins are so called due to their binding of the guanine nucleotides GDP and GTP. They exist as heterotrimers consisting of the three subunits α, β, and γ and are located at the inner surface of the plasma membrane associated with transmembrane G-protein-coupled receptors. Upon ligand binding, guanosine diphosphate (GDP) dissociates from the α-subunit and is replaced by guanosine triphosphate (GTP). Binding of GTP results in release of the βγ and α components, which in turn bind to and activate effector molecules (Wieland and Mittmann, 2003). Following hydrolyses of GTP by the α subunit, the hetrotrimer reassociates.

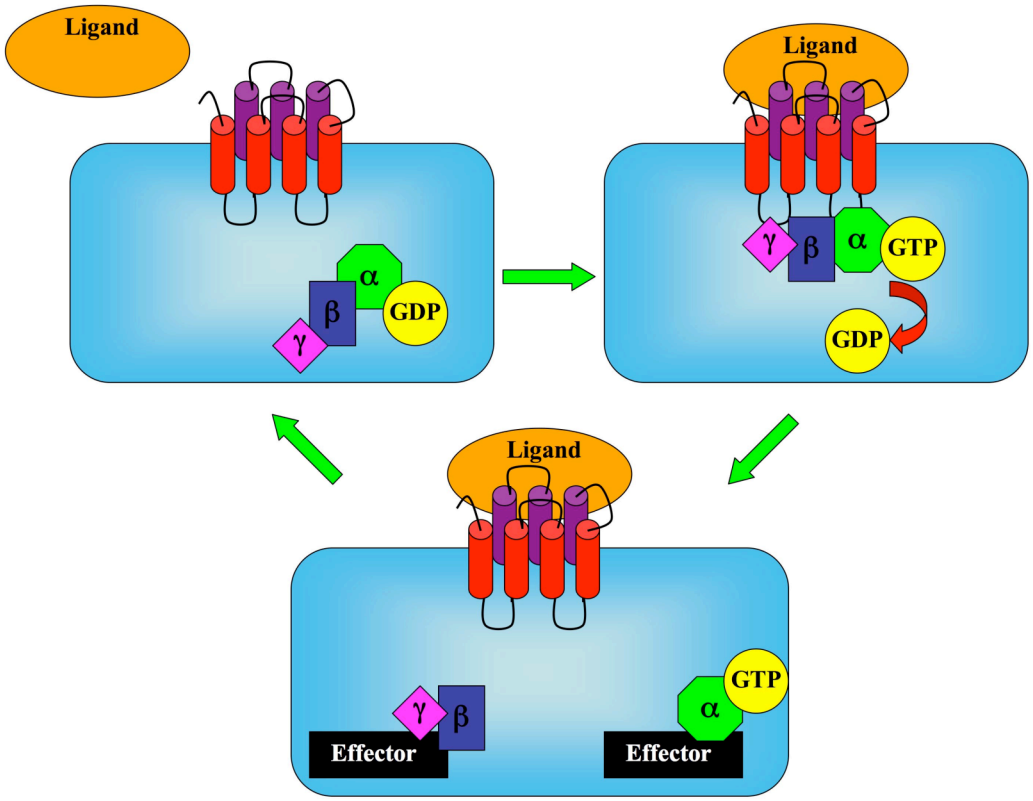


Figure 1.2.2: Schematic diagram depicting G-protein activation and hydrolyses (Colgan, 2006).

A 1996 study by Gudi *et al*. proved that activation of G-proteins in endothelial cells can occur as quickly as 1 s after the onset of shear stress, whilst Bao *et al*. (Bao et al., 2001) showed activation of Gαq/α11 and Gαi3/αo proteins was necessary for activation of downstream signalling cascades in endothelial cells. Moreover, antisense Gαq oligonucleotides can block shear induced Ras-GTPase activity (Gudi *et al*., 2003), whilst treatment of endothelial cells with pertussis toxin (a Gi inhibitor) prevented shear stress-mediated activation of ERK 1/2 (Jo *et al*., 1997).

Interestingly, the presence of the γ subunit at integrin-rich focal adhesion sites

has been reported (Hansen *et al*., 1994) - establishing a method not only for a single signal to activate two mechanotransduction pathways at once, but also implicating G-proteins in mediation of integrin activation. The work of Arcangeli *et al*. (Arcangeli et al., 1993) supports this hypothesis by proving that activation of potassium channels stimulated by integrin-dependent cell adhesion to the ECM can be blocked by G-protein inhibition.

1.2.1.5 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTK) are monomeric, transmembrane cell surface receptors composed of between 25-38 amino acids with extracellular N-terminal and intracellular C-terminal regions. Extracellular ligands include growth factors, cytokines and hormones, binding of which induces dimerisation with adjacent RTKs and rapid activation of intracellular (cytoplasmic) kinase domains. In turn the activated receptor is auto-phosphorylated on tyrosine residues found at the C-terminal region, creating binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing proteins (Pawson, 1995). Binding of these proteins results in their phosphorylation and activation with initiation of downstream signal transduction pathways (e.g. MAP Kinase pathway) (Zwick *et al*., 2001).

Approximately twenty different classes of RTKs have been identified, and in many cases both shear stress and cyclic strain implicated in their activation (e.g. exposure of SMCs to both hemodynamic forces results in activation and phosphorylation of PDGF receptor-α, (Hu *et al*., 1998). Flk-1 (a VEGF receptor and

RTK) is activated in response to shear stress (Zwick *et al*., 2001) and notably this effect is attenuated by the blockage of integrins (Wang *et al*., 2002).

1.2.1.6 Mechanotransduction Complexity

Mechanotransduction in response to hemodynamic forces constitutes a major area of ongoing research. While much is known about the mechano-sensors involved in signal transduction, many classes, sub-classes and families exist within each sensor, making elucidation of the components and downstream effects involved a lengthy process. Furthermore, interplay between sensors, such as those mentioned above (G-proteins and integrins, RTKs and integrins) adds to the complex nature of the interactions. Even more interesting still is the argument as to whether these molecules are themselves mechano-sensors or merely "mechanosensitive". It is entirely possible that a "primary" mechanosensor located elsewhere in the cell could detect hemodynamic forces and induce a signaling cascade resulting in the activation of the components mentioned above.

1.2.2 Endothelial Dysfunction and Cell Behaviour

Generally, "endothelial dysfunction" refers to dysregulation vessel homeostasis and nitric oxide/ ROS balance, with the aforementioned hemodynamic forces playing a central role in the process (Hayoz and Mazzolai, 2007). One can expand on this view and include a cell's most basic responses in this terminology. Endothelial

dysfunction is characterised by dysregulation of endothelial functional processes including proliferation, migration, apoptosis and angiogenesis. Moreover, hemodynamic imbalance is not the only reason for irregular cell function. The action of toxins, bacteria (Grandel and Grimminger, 2003), viruses, direct injury (Versari et al., 2007), genetic and dietary factors (van Boven et al., 1994) can all have a detrimental impact on endothelial health. It is pertinent therefore to examine endothelial cell behaviour in order gain a more complete understanding of its role in endothelial dysfunction.

1.2.2.1 Apoptosis

Apoptosis is a carefully regulated process of cell death that occurs as a normal part of human development and is distinguished from necrosis, or "accidental" cell death by characteristic morphological and biochemical changes. These include nuclear chromatin fragmentation and compaction, shrinkage of the cytoplasm and loss of membrane asymmetry (Allen *et al*., 1997, Darzynkiewicz *et al*., 1997, Lincz, 1998).

Ultimately responsible for apoptosis and its related alterations in cellular morphology are the caspase family of cysteine proteases that exist in their inactive forms within cells (Adams, 2003). Thirteen members have been identified of the human caspase family, which have been divided into two sub-groups. The members of the first sub-group function as initiators of the cell death process and include caspases 8, 9 and 10, while the second sub-group containing caspases 3, 6 and 7 act as effectors, cleaving death substrates that result in the cellular changes noted above.

In addition to caspases, the Bcl-2 family of proteins also regulate the process of apoptosis. The 18 identified members are divided into three sub-groups with the first comprising of Bcl-2 and Bcl-xL, both anti-apoptotic proteins. The second and third sub-groups contain Bax, Bak, Bid and Bad, all of which function in a pro-apoptotic manner.

Many stimuli are known to initiate the apoptosis pathway, such as death receptor expression, viral infection, ionizing radiation, T-cell recognition and also cellular stress. Activation via death receptors activates an extrinsic signalling pathway, while other stimuli, in particular cellular stress, result in intrinsic pathway activation. Both pathways converge to activate caspases (Danial and Korsmeyer, 2004).

Death receptors are located on the cell surface and transmit apoptotic signals intracellularly upon binding of their respective ligands, which include hormones, cytokines and cytotoxic T-cells displaying the enzyme granzyme. The receptors belong to the tumor necrosis factor (TNF) gene-superfamily and are capable of caspase activation within seconds of ligand coupling. The most characterised receptors are CD95 (or Fas), TNF receptor-1 (TNFR-1) and the TNF-Related Apoptosis Inducing Ligand (TRAIL) receptors DR4 and 5. Their actions are detailed in Fig. 1.2.3.

Apoptotic pathways result in the activation of the effector caspases 3, 6 and 7. They effect cellular chromosomal DNA, and alter nuclear structure and function in the following manner:

1) **Inactivation of enzymes involved in DNA repair:** Poly (ADP-ribose) polymerase (PARP), is involved in repair of DNA damage. Caspase 3 cleavage of the enzyme prevents its action.

2) **Inactivation of enzymes involved in cell replication:** Caspases inactivate DNA topisomerase II, an enzyme essential to DNA replication

3) **Fragmentation of DNA:** Caspase-activated DNase (CAD) is responsible for fragmentation of DNA into nucleosomal units. It exists in the cell in the form of the inactive ICAD; however, during apoptosis it is cleaved by caspases resulting in CAD release.

4) **Breakdown of structural nuclear proteins:** Lamins are vital to a cell's nuclear structural integrity. Caspase 6 degrades lamins resulting in chromatin condensation and nuclear fragmentation.

Endothelial monolayer homeostasis involves a balance between pro- and anti-apoptotic signals, with dysregulation of this balance central to the pathogenesis of vascular diseases (Mallat and Tedgui, 2000). Dysfunction of the apoptotic pathway resulting in increased cell death is thought to be responsible for conditions such as Parkinson's or Alzheimer's disease (Jellinger, 2001) and conversely a cell not undergoing apoptosis is the hallmark of a cancerous phenotype. Moreover, endothelial cell apoptosis is critical in pathological vascular remodeling (Ashton *et al*., 2005) and angiogenesis (Chavakis and Dimmeler, 2002). *In vitro* studies have shown that during the process of apoptosis endothelial cell adherens junction proteins are degraded with disruption of barrier function (Bannerman *et al*., 1998).

The same process *in vivo* would result in detachment of endothelial cells, with their subsequent removal into the bloodstream due to shear stress, leading to vascular leakage and exposure of the highly thrombogenic subendothelial matrix. This would point a role for apoptosis in inflammatory vascular diseases such as atherosclerosis (Stoneman and Bennett, 2004). Adding greater depth to this theory are studies by Bombeli *et al*., and Schwartz *et al*., who have shown apoptotic endothelial cells become proadhesive for platelets and leukocytes (Bombeli *et al*., 1999, Schwartz *et al*., 1999) and also pro-coagulant (Bombeli *et al*., 1997). Apoptosis is also thought to contribute to the pathogenesis of other diseases including heart disease (incubation of endothelial cells with serum from patients with heart failure induced apoptosis and downregulation of eNOS (Agnoletti *et al*., 1999)), Emphysema (endothelial apoptosis and progression of emphysema were reduced with administration of a caspase inhibitor in an experimental model (Voelkel and Cool, 2003)), and hypertension (endothelial cells became apoptotic in experimental hypertension in rats, (Suematsu *et al*., 2002)).

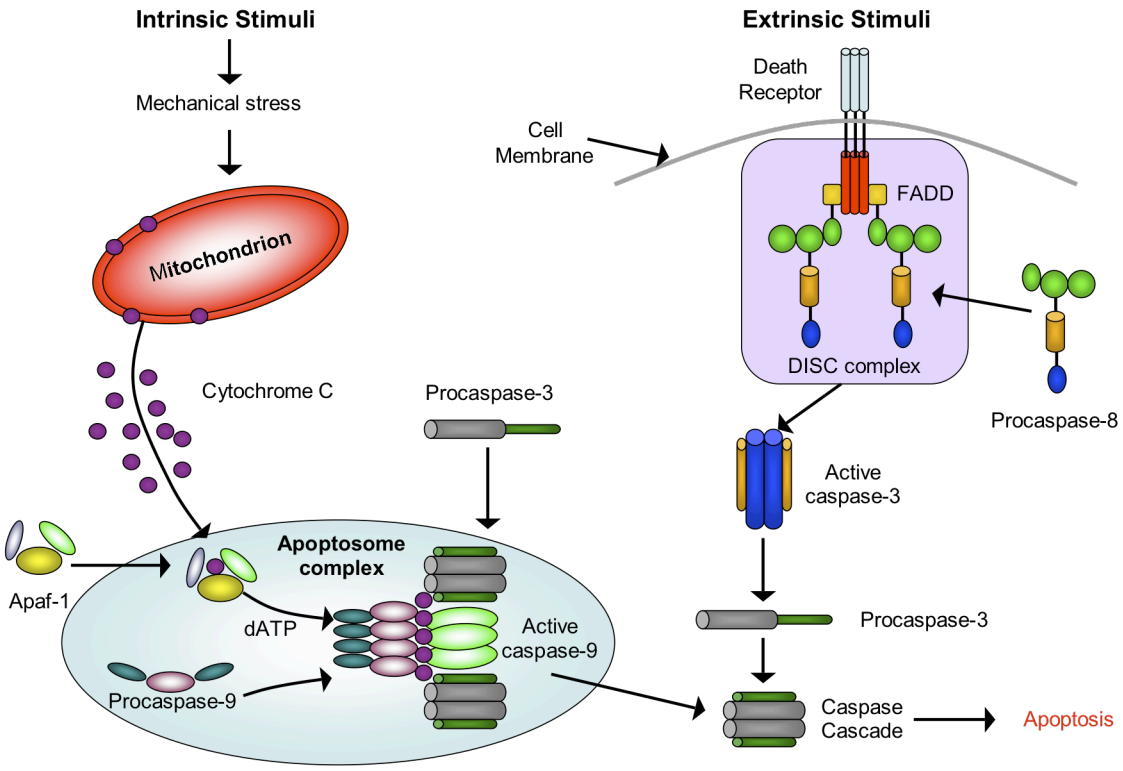


Fig 1.2.3 The effect of intrinsic and extrinsic stimuli on induction of apoptosis. Extrinsic binding of ligands to death receptors results in their trimerisation and clustering of the receptor's intracellular death domains. This allows binding of the adapter molecule Fas-Associated Death Domain protein (FADD) and procaspase-8 to the death domain forming a death-inducing signalling complex (DISC) and resulting in activation of the initiator caspase 8 (Chen and Goeddel, 2002). Activation of the intrinsic pathway due to cell stress results in mitochondrial perturbation and release of cytochrome c (whose regulation is controlled by the Bcl-2 family), which binds to apoptotic protease-activating factor 1 (Apaf1) (Salvesen and Renatus, 2002). This binding allows the formation of an Apaf1-caspase 9 complex resulting in activation of the initiator caspase 9.The activated initiator caspases 8 and 9, in turn activate the effector caspases 3,6 and 7.

1.2.2.2 Migration

Cell migration is a highly integrated process that plays vital roles in embryonic morphogenesis, tissue repair and regeneration, and contributes greatly to the advancement of diseases such as cancer, atherosclerosis and arthritis (Ridley *et al*., 2003). Although many aspects of the process are conserved between cell types, great variations can occur in the manner in which a cell migrates (Friedl and Wolf, 2003, Knight *et al*., 2000). The steps involved in endothelial cell migration- polarization, protrusion, traction and retraction, are outlined below.

1) **Polarization:** In order for a cell to migrate it must be polarized, meaning the molecular processes occurring at the front of the cell will differ from those at the back. Upon encountering a chemotactic agent, cells will organize into a frontal forward moving section and a rear-retracting portion. Establishment of this polarity along with its maintenance is governed by a set of inter-linked positive feedback loops involving integrins, microtubules, phosphoinositide 3-kinases (PI3Ks) and the Rho family of GTPases. In particular Cdc42 (Itoh *et al*., 2002), Par proteins and atypical protein kinase (aPKC) (Etienne-Manneville and Hall, 2003) initiate the polarization process. Cdc42 has been shown to localize the microtuble-organizing center (MTOC) and Golgi apparatus in front of the nucleus at the leading migratory edge (Etienne-Manneville and Hall, 2002, Rodriguez *et al*., 2003), both of which are cellular architectural changes known to accompany polarization. In order to respond directionally to a chemotactic stimulus, a small stimulus must be amplified to generate a large cellular response, central to this mechanism is production of phosphatidylinositol triphosphate (PIP_3). PIP_3 becomes rapidly produced by PI3Ks and localized to the front of a migrating cell, enhancing the polarization (Rodriguez *et al*., 2003).

2) **Protrusion:** The cell migratory cycle begins with the formation of a protrusion, with the actin cytoskeleton playing a central role in the process. Actin filaments are themselves polarized, driving membrane protrusion with rapidly growing "barbed" ends and slow-moving "pointed" ends. Protrusions can take the form of lamellipodia with a branching dendritic network of actin filaments, or filopodia where parallel bundles of filaments are formed. Actin polymerization in lamellipodia is controlled by the Arp2/3 complex, which is known to bind to the tip or side of actin filaments and promote formation of branching daughter filaments. Arp2/3 activation is induced by WASP/WAVE family members, which in turn are targets for Cdc42 and Rac. In contrast filopodia undergo protrusion in a tread-milling mechanism whereby actin filaments elongate at their barbed ends and dissemble at their trailing ends releasing actin monomers (Welch and Mullins, 2002).

3) **Traction:** In order for the cell to advance, attachment and stabilization of extended protrusions must occur to provide the cell with a means of traction to pull itself forward. Adhesions and integrins gather at the leading edge of the protrusion and provide this traction through ECM interactions. The tractional force required to physically move the cell is created at these adhesion sites by the contractile properties of myosin II combining with actin filaments and attached integrins (Beningo *et al*., 2001, Galbraith *et al*., 2002, Lauffenburger and Horwitz, 1996).

4) **Retraction:** To complete the cell migratory cycle, disassembly of adhesions at the rear of the cell and retraction of the cell's tail is necessary. As with the process of traction, myosin II plays an important role in developing a tension between the front and rear of the cell. Sufficient tension will open calcium-channels, resulting in activation of the protease calpain (Lee *et al*., 1999), which is responsible for cleavage of focal adhesion points, integrins talin, vinvulin and FAK at the rear of the cell.

The basic concepts surrounding cellular migration are well understood, however as with other cell processes, much research is still required to gain a greater understanding of all components of the process.

It is important to consider the role of endothelial cell migration in the context of vascular disease. Damage or injury to the endothelium resulting from chemical, mechanical and biological injury, or from perturbed blood flow has the potential to initiate a vascular pathology (Michalik and Wahli, 2006). In this respect, quick and efficient repair of the endothelium is vital in maintaining a healthy vasculature. An intricate series of events including inflammation, oxidative stress, immune cell recruitment, cell survival, proliferation, migration and differentiation, all act synergistically to repair the damaged tissue. Thus, uncontrolled migration (or indeed any of the aforementioned events) resulting in failed wound repair can have serious consequences including cell death and carcinogenesis. For example, decreased endothelial migration and hence wound repair, leaves highly thrombogenic sub-endothelial layers exposed, increasing the possibility for initiation of a vascular pathology (Versari et al., 2007). Moreover, an inflammatory phenotype would typically dominate in this situation, which has been shown as central to the formation of an atherosclerotic lesion (Barton *et al*., 2007).

1.2.2.3 Proliferation

The manner in which a cell proliferates is described by a cycle of events leading to cellular duplication and division- the cell cycle. It consists of four distinct phases: G_1 phase, S phase, G_2 phase (these are collectively known as interphase) and M phase, each of which are outlined in Fig. 1.2.4.

Regulation of cell cycle progression relies on two classes of molecules- cyclin-dependent kinases (CDKs) and cyclins. CDKs were first isolated by Paul Nurse in 1987, following his discovery of a corresponding gene (cdc2) in yeast. When bound to cyclin, CDKs become activated and phosphorylate target proteins involved in mediating entry into each phase of the cycle. Following a pro-mitogenic signal, G_1 cyclin-CDK complexes are activated and promote expression of transcription factors, which act downstream to promote expression of S cyclins and the enzymes involved in DNA replication. Moreover, the G_1 complex plays a role in degradation of molecules that inhibit the S-phase of cell cycle. The mitogen cyclin-CDK complexes, which promote initiation of mitosis, are synthesized during the S and G_2 phases. They act by stimulating production of the proteins involved in mitotic spindle assembly and chromosome condensation, and in the process, activate anaphase-promoting complex (APC). APC is a ubiquitin ligase known to degrade not only the mitotic cyclins (to allow cytokinesis to proceed), but also the structural proteins associated with the kinetochore (a protein which links the chromosome to the mitotic spindle microtuble polymers during mitosis).

Proteins that stimulate cell cycle are known are mitogens. They trigger the mitogen activated protein (MAP) kinase signaling cascade that regulates various

cellular activities such as gene expression, mitosis, differentiation and apoptosis (Pearson *et al*., 2001). MAP kinase activation in mammals occurs primarily through binding of growth factors to RTKs and subsequent signal transduction. Growth factors known to stimulate this pathway and initiate cell cycle in the endothelium include vascular endothelial growth factor (VEGF) (Guo *et al*., 1995), hepatocyte growth factor (HGF) (Wajih and Sane, 2003) and fibroblast growth factor (FGF) (Cross *et al*., 2000). The phases and various components involved in cell cycle are outlined diagrammatically below.

Similar to the effects of apoptosis, uncontrolled cell proliferation is implicated in disease states such as cancer, atherosclerosis, and intimal hyperplasia. Moreover, as proliferation plays an important role in vessel repair (Pandya *et al*., 2006), any reduction in its function leads to reduced wound closure following vascular injury. This can result (as with decreased migration) in initiation of an inflammatory or thrombogenic event and ultimately a disease state such as atherosclerosis. Conversely, increased proliferation in organ transplant recipients is considered central to organ rejection (Nickel *et al*., 2006), and much research has focused on treatments to prevent this (Chen *et al*., 2005, Lehle *et al*., 2005).

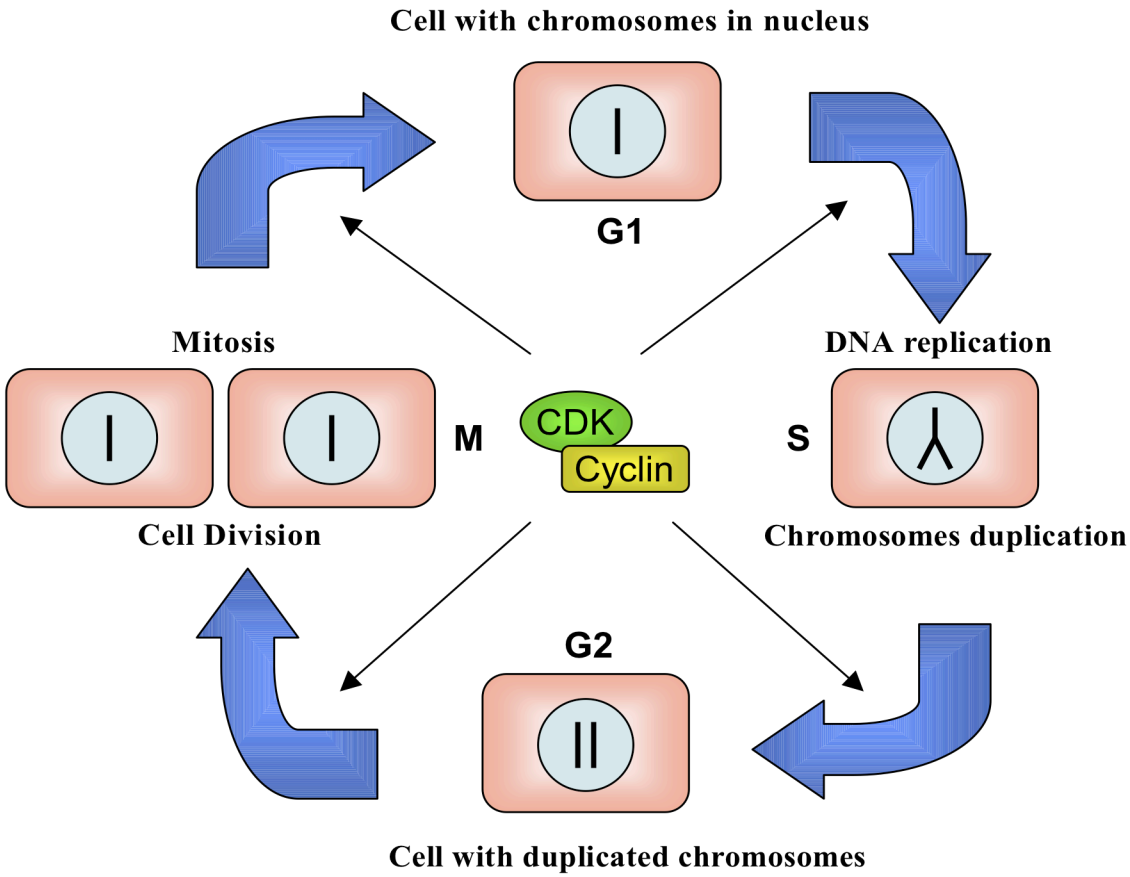


Fig. 1.2.4 The Cell Cycle. During the M phase of cell cycle the biosynthetic activities of the cell slow down considerably. These resume in the G_1 phase at a high rate and include production of RNA, protein synthesis and an increase in cell size. Next, in order to produce two daughter cells, two sets of DNA must be produced. This occurs during the S-phase. The cell then enters the G_2 phase where it continues to grow and produce new proteins. In the final M phase cells undergo mitosis, where the cell's chromosomes are divided between two daughter cells, and cytokinesis, in which the cell's cytoplasm divides. It is much shorter than interphase, lasting only 1-2 hours before the cell cycle begins again with the G_1 phase.

1.2.2.4 Angiogenesis

Angiogenesis is a natural process in the body that involves the growth of new blood vessels from existing vasculature (Folkman, 2003). It occurs in healthy individuals and is necessary for healing wounds and restoring blood flow to tissues after injury. Moreover in females, angiogenesis acts during the monthly reproductive cycle (in rebuilding the uterus lining and egg maturation during ovulation) and during pregnancy (to build the placenta and circulatory system between mother and fetus).

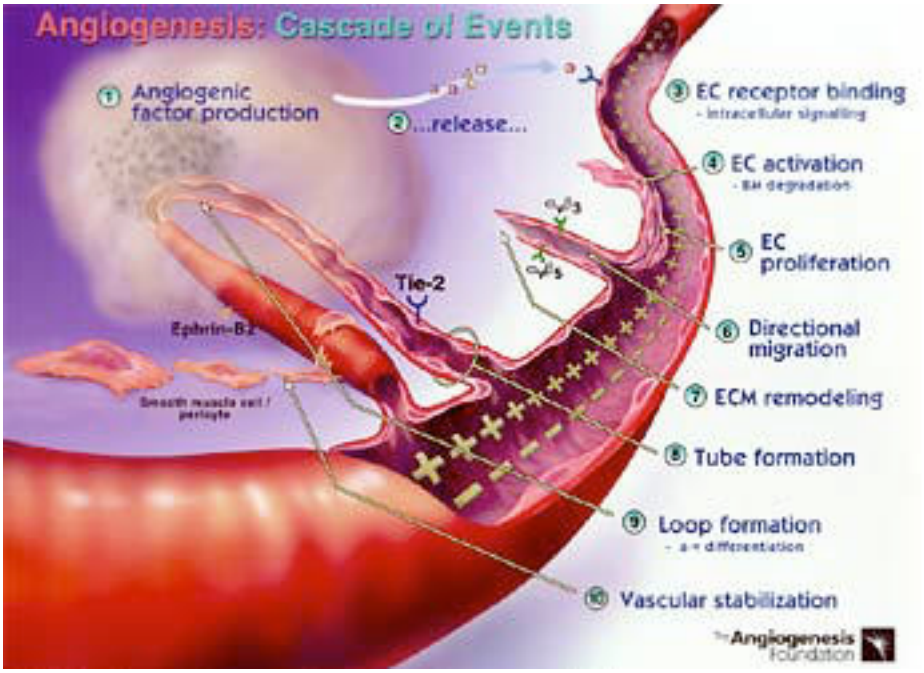


Fig. 1.2.5 The Angiogenic cascade. The above diagram shows the 10 main steps in the angiogenic cascade beginning with growth factor production and release, and resulting in formation and stabilization of a new blood vessel. (TheAngiogenesisFoundation, 2000)

The steps involved in vascular cell angiogenesis encompass all cell functions, beginning with proliferation; they are shown diagrammatically in Fig 1.2.5.

- In response to angiogenic stimuli, endothelial cells enter an actively proliferating state. Many molecules have been shown to participate in angiogenic stimulation including: VEGF (Goto *et al*., 1993), FGF (Klagsbrun, 1992), angiopoiteins (Thurston, 2003), platelet derived endothelial growth factor (PD-EGF) (Moghaddam *et al*., 1995) and hepatocyte growth facator (HGF) (Rosen *et al*., 1993).

- Once endothelial cells are activated they need to degrade the ECM to which they are attached. Under normal growth conditions the ECM provides necessary contacts between endothelial cells and the surrounding tissue, adding structural integrity to cells. Endothelial cells are encased in a basement membrane of collagen and laminin, and for the angiogenic process to continue, the activated endothelium produces potent proteinases to sever the attachments between cell and ECM. Proteinases produced include: plasminogen activators (such as urokinase plasminogen activator (uPA), MMPs, heparinases. chymases, tryptases and cathepsins (Jackson, 2002, Luttun *et al*., 2000, Pepper, 2001).

- Once free from the ECM, endothelial cells proliferate into the surrounding matrix and connect to neighboring vessels by forming solid sprouts. These protrusions respond and travel towards a chemotactic stimuli, which is achieved through cell migration with adhesions and integrins providing the necessary traction.

- Finally, loops are formed by the protruding sprouts that differentiate and become vessel lumen, blood vessel tubes connect to the sprouts to allow for circulation, and the vessel is given structural support by smooth muscle cells and pericytes (Montesano *et al*., 1993, Tille and Pepper, 2002, Velazquez *et al*., 2002, Villaschi and Nicosia, 1994).

Angiogenesis plays a major role in the pathogenesis of cancerous tumor formation. Tumors release VEGF to stimulate production of new blood vessels, which supply the nutrients necessary for tumor metastasis. Clinical experiments have now yielded the first drug aimed specifically at inhibiting tumor angiogenesis by preventing the action of VEGF using monoclonal antibodies (Ruegg and Mutter, 2007). As previously noted, maintenance of the endothelial monolayer and its functions are crucial for the prevention of cardiovascular disease (Versari *et al*., 2007). The process of angiogenesis is central to maintaining a healthy vasculature, in particular after vessel injury to repair and close a wounded endothelium. Known cardiovascular risk factors have been shown to impact on an endothelial cell's angiogenic ability- smoking (Su *et al*., 2004), high blood pressure (Sane *et al*.,

2004), and bacterial infection (Pearce *et al.*, 2004) all reduce endothelial angiogenesis. Thus, reduced endothelial repair can be viewed as an important early indicator of a cardiovascular disease state and the necessity for healthy angiogenic response to injury is clear.

1.2.3 Shear Stress and Endothelial Function

The effect of shear on endothelial function in the context of NO has previously been discussed, however many links have also been established between endothelial dysfunction and shear stress.

Laminar shear stress causes a dose-dependent reduction of endothelial cell proliferation (Levesque *et al.*, 1990) and a lower level of DNA synthesis over a sustained period, than cells growing under static conditions (Akimoto *et al.*, 2000). Conversely, perturbed shear stress results in a higher turnover rate of cells than those in static conditions (Davies *et al.*, 1986) and increased DNA synthesis (Chiu *et al.*, 1998).

Endothelial cell migration is enhanced during conditions of laminar flow (Sprague *et al.*, 1997, Wu *et al.*, 1995), and it is believed that this up-regulated directional migration in response to shear is related to lamellipodia and focal adhesion remodeling (Davies *et al.*, 1994, Li *et al.*, 2002). In turn, this remodeling is regulated by the Rho family of small GTPases (Wojciak-Stothard and Ridley, 2003).

Animal models have shown that endothelial cells exposed to perturbed shear have a junction permeability that is four times greater than that of laminar sheared cells (Schwenke and Carew, 1989). Moreover, endothelial tight junction proteins

ZO-1 and occludin are upregulated following shear (Colgan et al., 2007a).

A potent apoptotic suppression effect has been shown in cells exposed to physiological levels of laminar shear stress. This suppression is continued throughout exposure to pro-apoptotic stimuli such as TNF-α, oxygen radicals, oxidized LDL and serum depletion (Dimmeler *et al*., 1997). Furthermore, this effect can be attenuated by the inhibition of NO (Dimmeler *et al*., 1997). Laminar shear stress exerts an athero-protective effect on the endothelium; however as previously noted disturbed levels of shear have the opposite effect, with increased ROS production leading to an inflamed and pro-atherogenic cell phenotype (Cunningham and Gotlieb, 2005).

1.3 Atherosclerosis

The term cardiovascular disease refers to a number of blood vessel and arterial pathologies including cerebrovascular disease, coronary heart disease and atherosclerosis. Of these, atherosclerosis is perhaps the best studied, and much is known about the initiation and progression of the disease through both *in vitro* and *in vivo* research, making it an ideal experimental model.

Atherosclerosis is a complex, multi-step process involving many different aspects of vascular biology, with endothelial dysfunction playing a central role in the initiation and progression of the disease. Animal studies have provided much clarification of the events leading to and during atherosclerotic plaque formation, with apolipoprotein E knockout mice used most commonly in genetic and physiological studies (Tamminen *et al*., 1999).

As noted previously many factors are known to contribute to an atherosclerotic pathology. In regions of the arteries where disturbed flow predominates, such as arterial branching or curvature, endothelial cell shape and alignment differs greatly to that of cells under laminar flow. Perturbed flow regions show greater endothelial permeability to macromolecules such as low-density lipoprotein (LDL) and are preferential sites for initiation of atherosclerotic lesions (Gimbrone, 1999a). Moreover, a cell phenotype with increased ROS and decreased NO expression dominates in these areas, with marked endothelial dysfunction also present due to decreased mechanotransducer control and the influence of cardiovascular risk factors.

In these conditions, LDL accumulates in the sub-endothelial matrix and undergoes minimal oxidation by ROS. Oxidised LDL stimulates production of pro-inflammatory molecules such as adhesions (including ICAM, PCAM, VCAM, P-selectin and E-selectin), growth factors (e.g. macrophage colony stimulating factor), and chemotactic proteins (e.g. monocyte chemotactic protein-1, MCP-1). Furthermore, oxidised LDL can inhibit production of NO (Rosendorff, 2002), and increase ROS formation (Sukhanov *et al.*, 2006). Expression of adhesions (Frostegard *et al.*, 1991), chemoattractants and growth factors result in monocyte influx to the sub-endothelial layer, mediated in particular by ICAM and selectins (Collins *et al.*, 2000, Dong *et al.*, 1998). Monocytes "roll" along the endothelial surface and undergo firm adhesion to the cells, followed by diapedesis into the sub-endothelial layer through cell junctions. This action is governed by junction adhesion molecules (JAMs) (Chavakis *et al.*, 2004) with MCP-1 acting as a strong

chemoattractant.

Extensive modification of LDL in the sub-endothelial space results in highly oxidised LDL, which is rapidly taken up by macrophage, forming foam cells- so called due to their appearance under magnification. This heavy oxidation relies not only on ROS, but also on the action of numerous enzymes including myeloperoxidase (Podrez *et al*., 2000), sphingomyelinase (Marathe *et al*., 1999) and phospholipase (Ivandic *et al*., 1999), while the LDL uptake by macrophage is mediated by the scavenger receptors SR-A and CD36 (Febbraio *et al*., 2000, Suzuki *et al*., 1997). The death of foam cells releases extracellular lipids and other cell debris, which becomes a growing mass, forming a necrotic core in the atherosclerotic plaque.

Macrophages within the sub-endothelial space of the plaque, along with T-cells, secrete cytokines and growth factors that stimulate SMC migration, proliferation and extracellular matrix production. One such cytokine is expressed on macrophages and is termed CD40. Engagement of CD40 with its ligand CD40L results in the production of pro-inflammatory cytokines, adhesion molecules and matrix degrading enzymes (Schonbeck *et al*., 2000). Moreover, known cardiovascular risk factors, such as elevated homocysteine levels are known to contribute to the development of fibrous lesions by also stimulating SMC migration and proliferation (Gerhard and Duell, 1999). In this manner the sub-endothelial spaces becomes a milieu of enzymes, foam cells, SMCs (secreting extracellular matrices) and a necrotic core, giving rise to a complex, atherosclerotic plaque with a thin fibrous cap. The process of atherosclerosis is shown in Fig. 1.3.1.

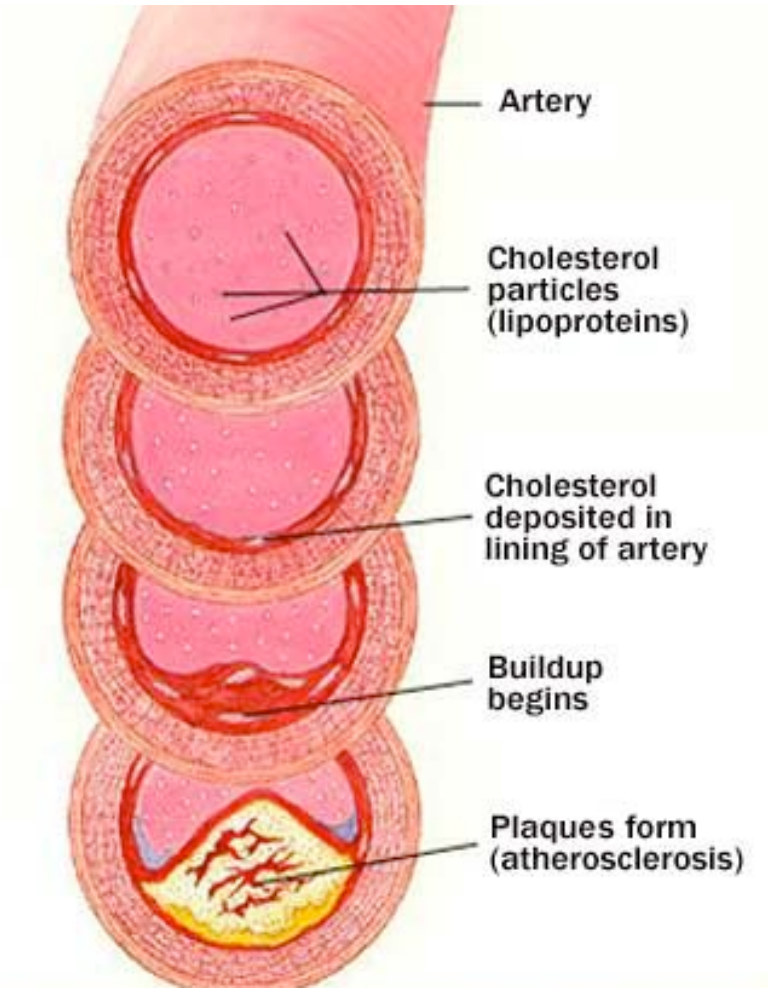


Fig 1.3.1. Progression of Atherosclerosis Over time, lipid accumulation, SMC migration and proliferation and matrix synthesis within the lesion narrow the arterial lumen, ultimately leading to myocardial ischemia. However, rupture of an atherosclerotic plaque is a far more serious matter, with the thrombogenic response capable of severely restricting blood flow to the heart resulting in myocardial infarction. (TheMayoClinic, 2000)

There are three main determinants of a plaque's vulnerability to rupture (Naghavi *et al.*, 2003): 1) The core size and consistency- a larger core of soft lipid-rich atheromatous gruel is highly unstable. 2) Thickness of the fibrous plaque- cap thinning increases a plaque's ability to rupture. 3) Cap inflammation and repair- a

cap that is slow to heal due to decreased SMC presence, or an inflamed cap due to macrophage influx is more likely to rupture.

Much progress has been made in identifying the factors contributing to the formation of an atherosclerotic lesion and in particular the risk factors involved in the disease. Hence, it is important to note that shear stress is not the only factor involved. *Smoking* has been shown to decrease HDL whilst increasing LDL cholesterol levels (Dautzenberg, 2005), *obesity* also results in higher cholesterol levels and increases blood pressure (Bray and Bellanger, 2006) and *increased homocysteine levels* have been shown to damage the arterial lining as well as promoting the formation of blood clots (Castro *et al*., 2006). Moreover, research has yielded an ever-increasing list of these factors including depression and other behavioural traits (Glassman and Shapiro, 1998), systemic inflammation (Kugiyama *et al*., 1999) and metabolic syndrome, along with the other risk factors of cardiovascular disease previously referred to. Non-traditional factors are also being explored with many promising results, in particular the role of infectious diseases in initiation and pathogenesis of atherosclerosis appears plausible. The oncogenic, Gram-negative bacterium, *Helicobacter pylori* (*H. pylori*), is one such pathogen.

1.4 *Helicobacter Pylori*

In 1875 when German scientists discovered a spiral shaped bacterium colonizing the mucus layer of the human stomach, an inability to grow the bacteria meant their findings were forgotten and ignored (Blaser, 2005). Subsequent references to this "spiral bacteria" by Giulio Bizzozero (1892) and Professor Walery Joworski (1899) also went largely unnoticed (Konturek, 2003). However in 1984 our understanding of *Helicobacter pylori* began in earnest as Nobel Prize winners Robin Warren and Barry Marshall suggested that gastric "curved bacilli" were associated with gastritis and peptic ulceration (Marshall and Warren, 1984). Our knowledge on this bacterium is now vast with over 1,700 publications a year for the last decade. Outlined below is our current understanding of the important aspects associated with *H. pylori.*

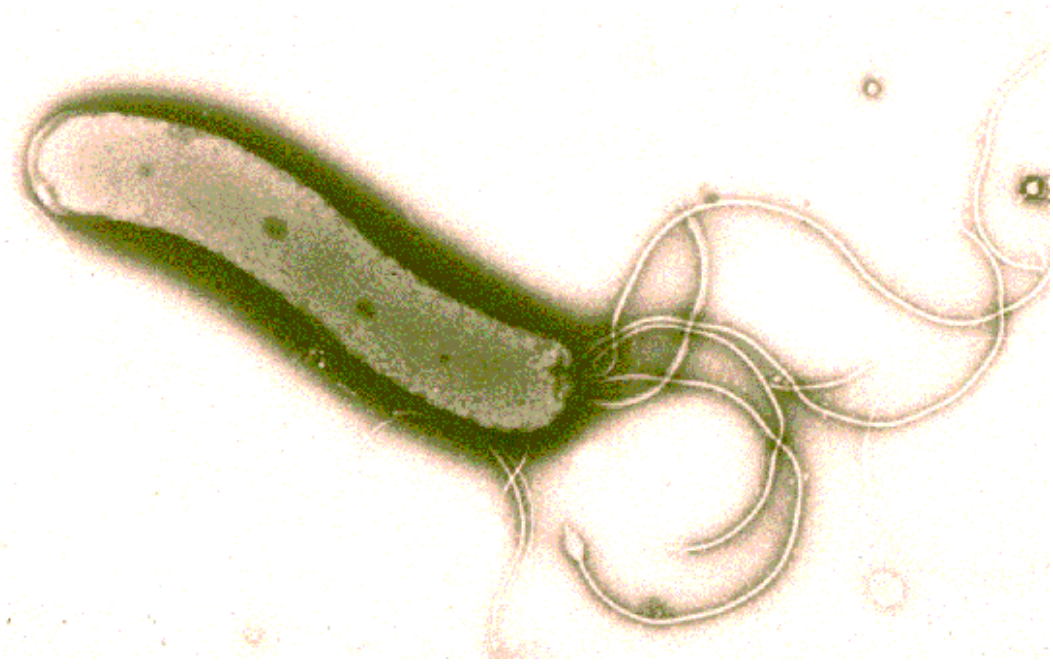


Fig. 1.4.1. *Helicobacter pylori* under high magnification. (Kelly D. J., 2006)

1.4.1 Characteristics and Epidemiology

H. pylori is a Gram-negative, micro-aerophillic, spiral-shaped, bacterium that colonises the human gastric epithelium and is strongly implicated in chronic gastritis, peptic ulcer disease and gastric carcinoma (Dunn *et al.*, 1997, Hatakeyama, 2004, Kuipers, 1997, Suerbaum and Michetti, 2002). The bacterium is about 3 μm long and 0.5 μm in diameter with 4-6 flagella, and is the only known bacterium to thrive in the harsh acidic conditions of the stomach. Utilizing the enzyme urease, *H. pylori* converts urea- of which there is an abundant supply, into bicarbonate and ammonia, thus neutralizing any acid in the surrounding area and providing the bacteria with optimum pH neutral growth conditions at 37°C. It produces two major virulence factors, Cytotoxin-Associated Gene A (CagA) and Vacuolating Toxin A (VacA), which are responsible for the many deleterious effects associated with the bacteria (Ernst *et al.*, 2006, Radosz-Komoniewska *et al.*, 2005).

The prevalence of *H. pylori* varies according to numerous factors including geographic location, race, age, sex and socio-economic status. However, greater levels of infection are found in developing countries with emerging economies (typically 80-90% of the population will be *H. pylori* positive) as opposed to developed countries with a strong economy (approximately 10% will test positive) (Frenck and Clemens, 2003, Parsonnet, 1995). Of those infected, only 10-15% will ever require medical treatment with 1% progressing to adenocarcinoma and fewer experiencing gastric mucosa-associated lymphoid tissue lymphoma. Irish figures for infection reveal the overall prevalence for *H. pylori in* Northern Ireland was as high as 50.5% in 1997. These figures are well outside the expected ranges for a

developing country. Numerous risk factors for *H. pylori* infection have been explored and their relevance evaluated: a link between *smoking* (Lin *et al*., 1998) and the bacteria does not appear likely based on the literature; the anti-microbial effect of *alcohol* (Brenner *et al*., 1997) may reduce the risk/severity of *H. pylori*; and the preparation of *food* in unsanitary conditions may be a mode of transmission for the bacterium. In addition *water-borne exposures* (Zhang *et al*., 1996)*, hygiene practices* (Goodman *et al*., 1996) such as sharing a toothbrush or cup with an infected individual, *high population density/overcrowding* (Hammermeister *et al*., 1992) and a *family history of gastric disease* (Brenner *et al*., 2000) have all been implicated as *H. pylori* risk factors. Transmission of the bacteria may occur through: person-to-person contact (i.e. saliva, vomitus, feces); contaminated drinking water (*H. pylori* may live days in milk or tap water); from animals such as cats or pigs; and the use of endoscopy techniques to examine gastric disorders (endoscopes can be difficult to disinfect due to their complex shape).

1.4.2 Genome and Genetic Diversity

The publication of two complete genomic sequences for *H. pylori* strains 26695 and J99 (Alm *et al*., 1999, Tomb *et al*., 1997), provided valuable information regarding the toxins, lipoproteins, and adhesions present in the bacteria along with details on well developed systems for motility, scavenging iron and DNA restriction and modification. Importantly it was also shown that *H. pylori* is likely to use recombination and slipped-strand mis-pairing within repeats as mechanisms for antigenic variation and adaptive evolution. This is based on the large number of

sequence-related genes encoding outer membrane proteins and the presence of homopolymeric tracts and dinucleotide repeats in coding sequences- traits displayed by other diverse bacteria. This would in part explain how *H. pylori* could remain alive in the human body for the duration of an individual's life causing chronic gastritis and gastric lymphoma.

1.4.3 Secretions

Once present in the stomach, the secretions of *H. pylori* are vital for its survival and successful colonization of the gastric epithelium (Andrutis *et al*., 1995, Eaton *et al*., 1991, Pugsley, 1993). These secretions enable the bacteria to fight the harsh acidic conditions of the gastric mucosa, and are known to contribute greatly to the pathogenesis of *H. pylori*-related diseases. Factors released such as VacA have been shown to significantly effect not only cellular morphology, but also cell processes such as apoptosis, proliferation, angiogenesis and migration (Blaser, 1992, Crabtree *et al*., 1991, Pearce *et al*., 2004). Secretions such as urease and heat shock proteins, which also have confirmed roles in gastric cell function, are confined exclusively to the cytoplasm of other bacteria, yet they are released into the extracellular space by *H. pylori*. This has led to studies of the mechanisms by which the bacterium secretes its known virulence factors, proteins and enzymes. Three such secretion methods have been identified:

1) A specific, selective release of proteins including VacA- through a type V secretion system, and CagA- injected into the host cells by means of a type IV secretion system (Cui and Shao, 2007, Nguyen *et al*., 2001).
2) Non-selective entry of *H. pylori* proteins through bacterial autolysis, (this was concomitant with gradual decrease in the optical density and bacterial viability in broth cultures studies). Moreover, autolysis accounts for the surface localization of proteins such as urease and heat shock protein B (HspB). A detailed description of which is outlined below (Fujita *et al*., 2005).
3) Many extracellular proteins are thought to be released in membrane vesicles or blebs, with several studies supporting this hypothesis (Fiocca *et al*., 1999).

Of these mechanisms, bacterial autolysis accounts for the bulk of secretion, whilst to Phadnis *et al.* propose it as a method for expression of cytoplasmic proteins on the bacterial cell surface (Phadnis *et al*., 1996). Urease and HspB are examples of such proteins, and are strictly expressed in the cytoplasm of *H. pylori* during log phase growth. The mechanism of autolysis is presented in Fig. 1.4.2.

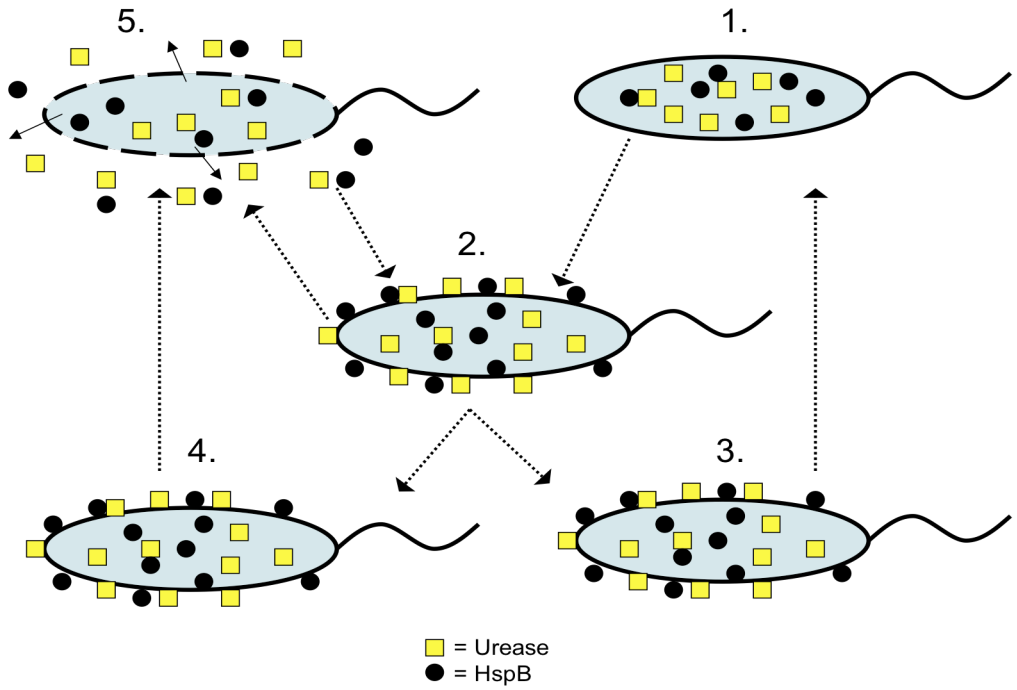


Fig 1.4.2 Autolysis in *H. pylori*. 1) Autolysis is thought to occur under appropriate environmental conditions (such as favourable pH, temperature and nutrient availability), releasing proteins along with putative virulence or mutagenic factors. 2) Adsorption of Urease and HspB onto the surface of remaining intact bacteria follows, at which point the bacteria can either 3)/ 4) divide to form daughter cells (with the surface absorbed proteins intact), or 5) undergo autolysis.

Autolysis and subsequent adsorption has other functions during pathogenesis of *H. pylori*-associated disease:

- Release of many antigenic factors at once overwhelms the immune system and makes evasion of host defences easier. Similarly, intrinsic outer membrane proteins can be masked from the immune system by the presence of adsorbed urease and HspB.

- It allows the bacteria to present virulence factors and antigens to the gastric mucosa and immune system *in vivo* (significant humoral responses are induced against Urease and HspB).
- It likely influences the specificity and magnitude of the interaction of *H. pylori* and gastric cells.

Supplementary to the knowledge on the mechanisms involved in secretion is the contribution made by numerous studies on the identification of the released factors. Schraw *et al.*, Kim *et al.* and Cao *et al.* all conducted such experiments focusing on proteins released in culture broth by *H. pylori* (Cao *et al*., 1998, Kim *et al*., 2002, Schraw *et al*., 1999). The proteins identified along with their function and known effects are outlined below.

1.4.3.1 Vacuolating Cytotoxin A

Five secretions systems (Type I-V) have been characterised for Gram-negative bacteria (Saier, 2006). Briefly, Type I Protein Secretory Pathway exporters (ISP) belong to the ATP-binding cassette ABC superfamily, of which 65 members are known. Two cytoplasmic domains that hydrolyze ATP molecules and two integral membrane channel-forming domains feature in this pathway. Transport is limited by the size and ease of unfolding of the protein being exported. Type II pathways (IISP) involve a two-step mechanism of transport, and are responsible for the export of numerous proteins across the cytoplasmic membrane by means of a general secretory (Sec) system (through the main terminal branch of the general secretory

pathway). Type III one-step systems are associated with pathogenicity in Gram-negative bacteria and allow secretion of cytoplasmically synthesized proteins across the cell membrane. Type IV systems are termed conjugation and virulence related pathways (IVSP) and consist of a number of subunits that span the membrane of the Gram-negative bacterial cell. They are capable of transporting proteins and DNA from the bacterial cell into the cytoplasm of the recipient cell (e.g. *Bordetella pertussis*). Finally, the Type V secretion system, often referred to as a sub-section of IISP, (due to its use of the Sec system) is an auto-transporter system (Henderson *et al*., 2000). VacA secretion falls into this last category and is examined below.

Following the discovery of *H. pylori,* it was noted that a protein present in broth cultures could induce formation of large vacuoles in cultured mammalian cells- the vacuolating cytotoxin A protein, (encoded by a chromosomal gene known as VacA) was found to be responsible (Leunk *et al*., 1988, Cover and Blaser, 1992). VacA is made up of an A-subunit that has enzymatic activity and a B-subunit that is responsible for recognising and binding to receptors on the target cell surface. Thus, it falls into the category of an AB group toxin. It is synthesized as a 140 kDa pro-toxin, which is cleaved at both the N-terminal signal sequence and C-terminal domain to yield an 88 kDa mature toxin, which is secreted into the extracellular space via a Type V auto-transporter secretion system (Telford *et al*., 1994, Nguyen *et al*., 2001) - this process is represented in Fig 1.4.3. Reyrat *et al.* demonstrated that the mature toxin is then proteolytically cleaved resulting in a 55 kDa C-terminal fragment, which binds to host cell receptors. This is then followed by internalisation of the remaining the 33 KDa N-terminal fragment, leading to vacuolation (Reyrat *et*

al., 1999). The structure of the 140 kDa protoxin along with its proteolytic processing and secretion is characteristic of other Gram-negative bacterial auto-transporter proteins. Apart from the aforementioned N-terminal signal sequence (or leader sequence), these characteristics also include a region that becomes the mature cytotoxin (often termed a passenger domain- the 88 kDa fragment secreted toxin noted previously), and a C-terminal domain with the ability to form β-strands, which aid translocation by inserting into in outer membrane in a β-barrel structure (Henderson *et al*., 2000).

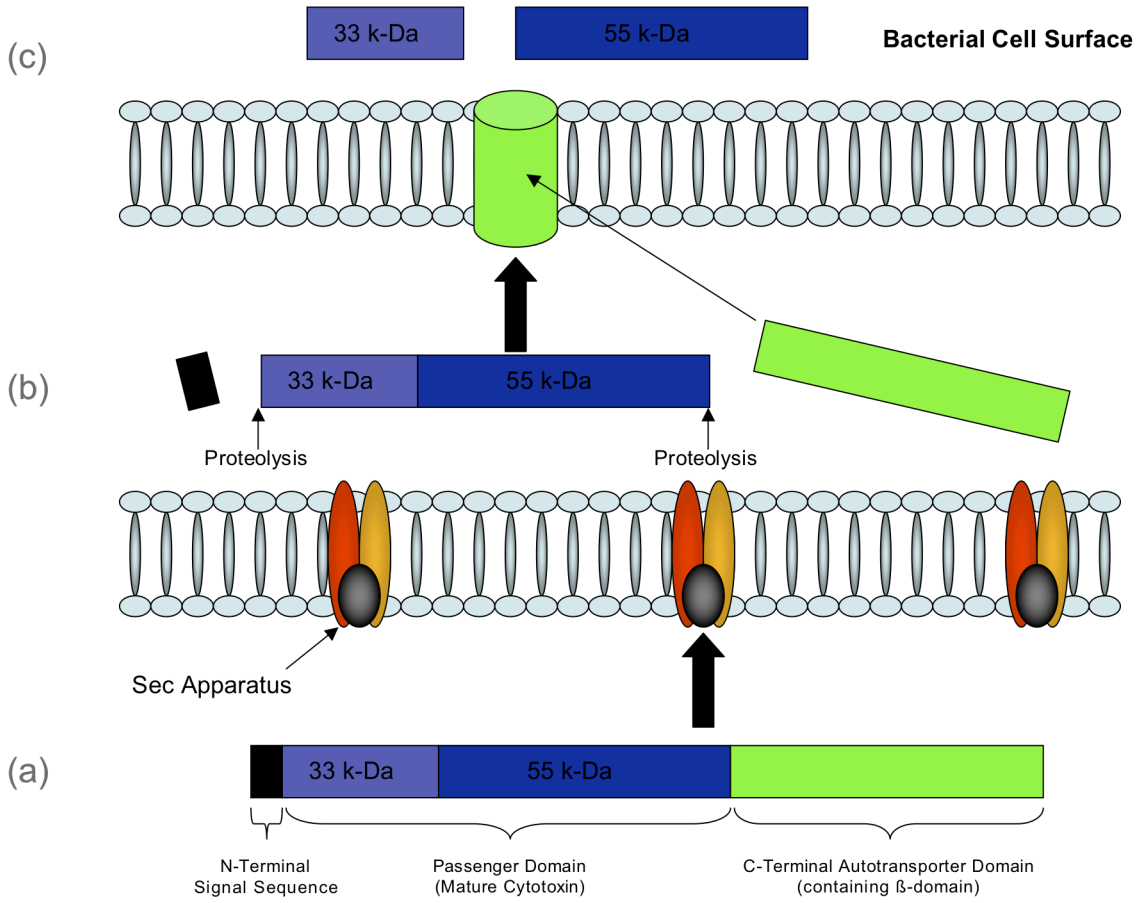


Fig 1.4.3. The Autotransporter System of *H. pylori* (a) 140 KDa protein is synthesised and exported through the bacterial cytoplasmic membrane via Sec machinery. (b) Once through the membrane, the signal sequence is cleaved and the c-terminal β-domain inserts into the outer membrane of *H. pylori* in a barrel structure, forming a pore through the membrane. (c) The passenger domain is then inserted into this pore and is translocated to the bacterial cell surface for further proteolytic cleavage.

It is important to note here that much diversity exists between VacA alleles of differing *H. pylori* strains and is at its most prominent in two distinct regions of the gene- namely the signal sequence region (s-region) and, in the middle of the passenger domain (mid region) (Fig 1.4.4). Two distinct sequences have been identified in the s-region, S1 and S2. Similarly, differing M1 and M2 sequences are found in the mid region. Strains containing the gene sequence for S1/M1 toxins are far more virulent, (and more widely studied) than those of S2/M1 or S2/M2 (Atherton *et al.*, 1997, Atherton *et al.*, 1995).

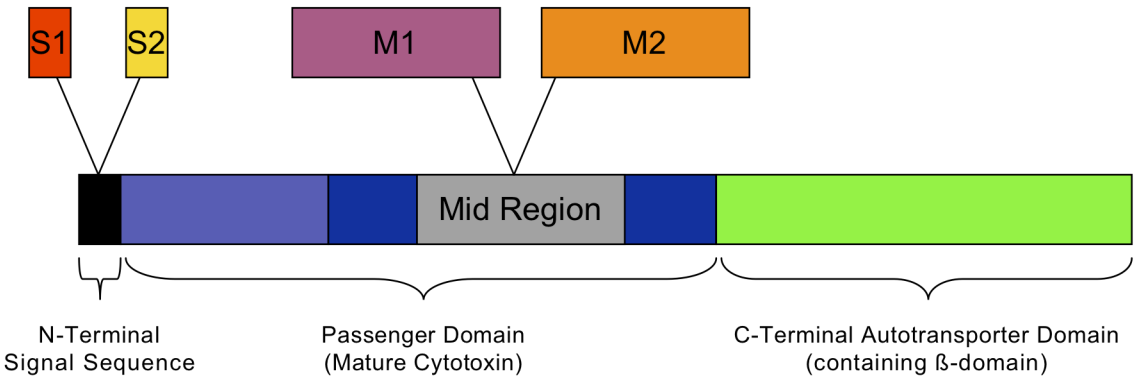


Fig 1.4.4 Genetic Diversity in *H. pylori* The cleavage site for S1 and S2 sequences is not the same, resulting in an extra 12 amino acid hydrophilic extension in S2 alleles. Only when this extension is absent will VacA exert its vacuolating activity. Reciprocally, when the toxin includes an M1 sequence, it's vacuolating effects are greater than with M2.

Once secreted from the bacteria the mature 88 kDa VacA toxin exists as a monomer capable of oligomerising to form a six- or seven-sided flower-like structure approximately 12 nm in height and 30 nm in diameter. Under acidic pH, it returns to its monomer form, an action associated with marked increase in

vacuolating activity (Cover, 1998). VacA is classified as a multi-functional toxin, capable of binding to many receptors and as such interactions of the toxin with host cells are poorly understood. It has been shown to bind to various lipids (Moll *et al*., 1995, Molinari *et al*., 1998, Czajkowsky *et al*., 1999), epidermal growth factor (EGF) receptor (Seto *et al*., 1998), heparan sulphate (Utt *et al*., 2001), RTPTα and RTPTβ (Yahiro *et al*., 2003, Padilla *et al*., 2000). The latter remains the best described.

Phosphorylation of proteins on tyrosine residues is necessary for transmission of the signals responsible for cell growth, proliferation and differentiation. Protein tyrosine phosphatases (PTPs) in conjunction with protein tyrosine kinases (PTKs) regulate this phosphorylation. While much is known on the subject of PTKs, little characterisation has been performed on PTPs, however basic information is available. PTPs are classified into two main groups- cytoplasmic proteins and transmembrane receptor proteins (Johnson and Van Vactor, 2003, Bixby, 2001). These receptor proteins (RPTPs) bear a resemblance to cell adhesion molecules and are hence believed to function in cell-cell communication. RTPTs comprise of an extracellular and transmembrane domain along with a cytoplasmic portion that contains one or two tyrosine phosphatase domains. RTPTβ is distinct from other RTPTs as a sequence within its extracellular domain bears homology to the enzyme carbonic anhydrase (Barnea *et al*., 1993).

Cells resistant to vacuolation by VacA become sensitized when transfected with plasmids encoding RTPTβ. In addition, VacA's cytotoxic effects on AZ-521 gastric cells is inhibited with RTPTβ antisense oligonucleotides (Schraw *et al*.,

2002, Padilla *et al*., 2000). This data suggests that binding RTPTβ is necessary or at least has an important role, for host cell vacuolation. Other known effects of VacA binding of RTPTβ include activation of the signalling pathways resulting in Git 1 tyrosine phosphorylation (Git 1 is involved in pathways responsible for growth factor signal transduction), and detachment of primary murine gastric epithelial from a reconstituted basement membrane (Fujikawa *et al*., 2003).

Much remains to be learned about binding of VacA to host cells and of the receptors involved. Complicating the issue, S1/M1 strains of *H. pylori* have different binding patterns and target different receptors in comparison to S2/M2 bacterial strains. Moreover, the targeted cell surface receptors may vary from one cell type to the next (Ji *et al*., 2000, Pagliaccia *et al*., 1998, Wang *et al*., 2001).

1.4.3.1.2 Cellular Effects Associated with VacA

1) Vacuolation: The most recognisable effect VacA on cells is the formation of vacuoles throughout the cell body. Following binding of VacA to the cell surface, it is internalised into the cell and formation of anion-selective VacA channels in the membranes of late endocytic compartments occurs (Tombola *et al*., 1999, Szabo *et al*., 1999, Morbiato *et al*., 2001, Czajkowsky *et al*., 1999). Due to increased chloride conduction through these channels, intra-luminal chloride concentrations are increased and to compensate, vacuolar ATPase activity rises, resulting in an upsurge in proton pumping and a reduction in intra-lumenal pH. Weak bases such as ammonia diffuse and become trapped in the late endocytic compartments, with

subsequent osmotic swelling of these compartments leading to vacuolation. Interestingly, vacuolated cells exclude trypan blue (Leunk *et al*., 1988), indicating that the process is not lethal to the host cell.

2) Mitochondrial Effects: In addition to VacA's presence within cell membranes, several studies report mitochondrial localisation (Willhite and Blanke, 2004, Galmiche *et al*., 2000). VacA was shown not only to decrease mitochondrial membrane permeability, but also to stimulate cytochrome C release- a process leading to caspase-3 activation and cell apoptosis (Willhite *et al*., 2003, Kimura *et al*., 1999). Concomitantly, a reduction of cellular ATP concentrations and impaired cell cycle progression has been shown following exposure to VacA (Kimura *et al*., 1999). Notably NPPB (5-nitro-2- (3-phenylpropylamino) benzoic acid) - which prevents the formation of vacuoles by blocking the chloride channels through which VacA operates, inhibits the aforementioned mitochondrial effects (Willhite *et al*., 2003, Willhite and Blanke, 2004).

3) **Signal Transduction Effects**: VacA induces activation of MAP kinases p38 and ERK1/2 in conjunction with the activating transcription factor 2 (ATF2) signalling pathway. However, this activity has no effect on vacuole formation or cytochrome C release- indicating that VacA activation of the p38 pathway is independent of these (Nakayama *et al*., 2004). As of yet, no downstream function of this activation has been assessed and no surface receptors leading to their activation have been identified.

4) **Paracellular Permeability Effects**: Papin *et al.* have shown a decrease in transepithelial electric resistance (TER) owing to an increase in paracelllular

permeability to low molecular mass molecules following exposure to VacA (Papini *et al*., 1998). Again, mechanisms involved in this effect are not well understood.

From its secretion method to cell surface interactions and subsequent effects on cell function, much remains unclear about *H. pylori* VacA. Recent studies have shown similar effects in vascular endothelial cell lines (Jenkinson *et al*., 2002, Pearce *et al*., 2004), suggesting a putative role in vascular disease and in particular, endothelial dysfunction. However this hypothesis requires much experimental exploration and consolidation.

1.4.3.2 Cytotoxin Associated Gene A

The CagA gene of *H. pylori* is recognised as a marker for increased risk of peptic ulcer disease and gastric cancer when present in bacterial strains (Blaser and Crabtree, 1996, Kuipers *et al*., 1995, van Doorn *et al*., 1999). Early research into differences between variant *H. pylori* strains and isolates led to the association of CagA- positive status with increased pathogenicity and the ability to induce numerous morphological and biochemical changes in human gastric cells (Leunk *et al*., 1988). The gene is present in 50 to 70% of strains (Ching *et al*., 1996, Dore *et al*., 2001) and is located at one end of the Cag pathogenicity island (PAI), a 40 bp DNA segment, which encodes 31 genes (Akopyants *et al*., 1998, Censini *et al*., 1996, Covacci *et al*., 1993). Eighteen of these genes are involved in the formation of the Type IV secretion system (T4SS), whose syringe-like structure is capable of penetrating and translocating bacterial macromolecules into the host cell (Asahi *et*

al., 2000, Christie and Vogel, 2000). The CagA protein (encoded by the CagA gene) is one such macromolecule, and acts as a substrate for the T4SS.

As previously mentioned, the Type IV secretion system is one of 5 such systems that exist in Gram-negative bacteria. It has a generic role in transporting toxins and DNA or protein-DNA complexes to host cells. The T4SS of *Agrobacterium tumefaciens* is the most studied model (Li *et al*., 1998), and comparison to the *H. pylori* system reveals a homologous structure, which is outlined in detail below. It consists of three distinct sub-structures: the coupling protein (CP) homo-multimer; a transenvelope protein complex; and the conjugative pilus. Mating-pore-formation (Mpf) proteins are assembled from the latter two substructures (Fischer *et al*., 2001). The three structures most likely act cohesively as one supramolecular organelle to mediate the stages of translocation: 1) Recruitment of DNA and protein substrates to the transfer machine; 2) Transfer of substrates across the cell envelope; and 3) Delivery to target cells. The function of each element of the T4SS is as follows: (Fig. 1.4.5)

Coupling protein:

VirD4 - recruitment of DNA and protein substrates to the Mpf complex (VirB1-VirB11);

Energy components:

VirB4 and B11 - ATPase homomultimer, provides energy for substrate export and pilus biogenesis.

Channel subunits:

VirB3 - function unknown.

VirB6 - assembly factor.

VirB8 - assembly factor and bridge between subcomplexes.

VirB9 - outer membrane pore signal peptide, which is cleaved by peptidase.

VirB10 - bridge between inner and outer membrane subcomplexes.

T pilus:

VirB2 – A cyclized pilin subunit signal peptide that is cleaved by peptidase. It starts as inner membrane protein until the periplasmic end is cleaved and undergoes cyclization and polymerization to form the T-pilus.

ViB5 – A pilus subunit chaperone.

VirB7 - A lipoprotein involved in pilus assembly.

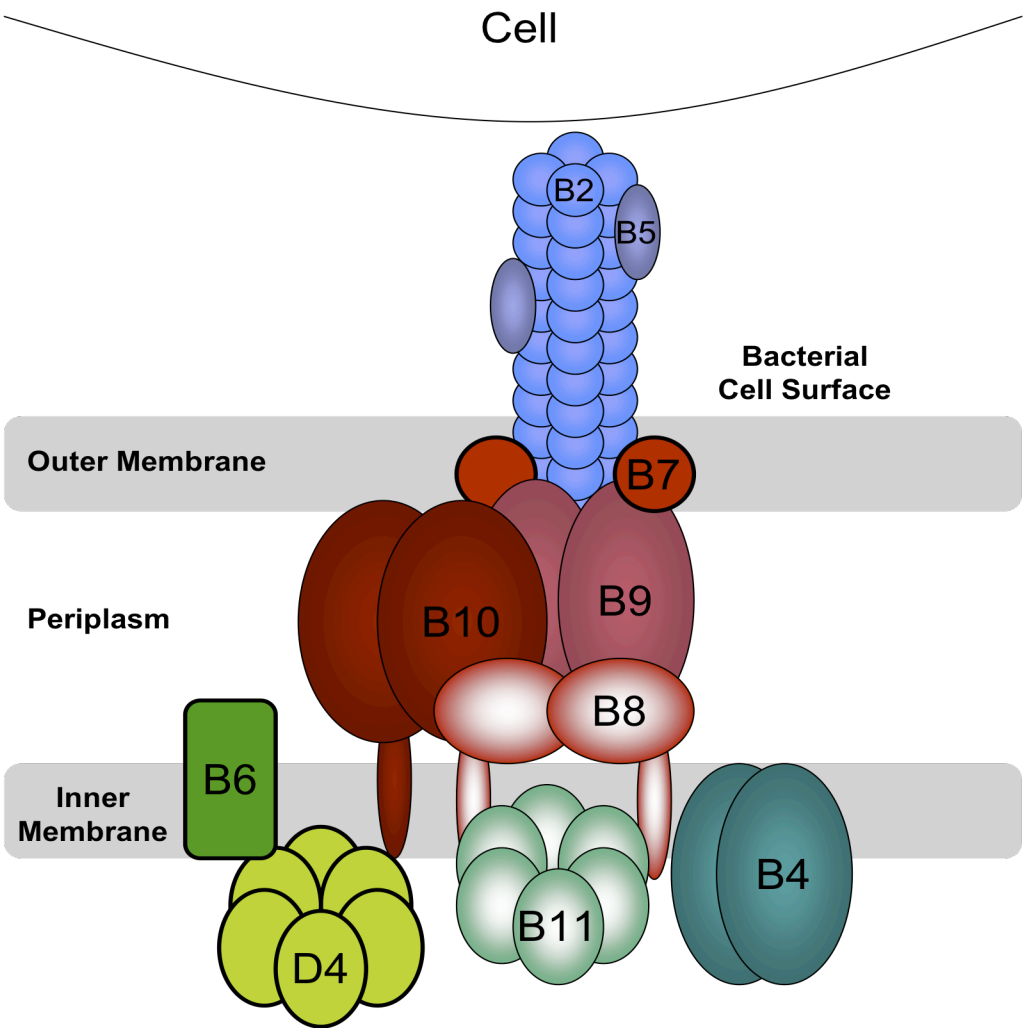


Fig. 1.4.5 The Type IV Secretion System

Once translocated into the endothelial cell the CagA protein is known to interact with numerous pathways and to stimulate a host of cellular responses. Upon injection, the vast majority of the protein (approximately 80%) localizes at the plasma membrane and undergoes tyrosine phosphorylation at the CagA EPIYA motif (a five amino-acid sequence that is present in the carboxy-terminal variable region of the protein) by the host cell Src family protein tyrosine kinases (SFK)

(Selbach *et al*., 2002, Stein *et al*., 2002). Once phosphorylated, membrane integrated CagA then binds to a cytoplasmic SRC homology 2 domain (SH2), which contains a protein tyrosine phosphatase (PTP) called SHP2 (Feng and Pawson, 1994). This results in a conformational change of SHP2 and continuous PTP activation (Hatakeyama and Higashi, 2005).

CagA-activated SHP2 can stimulate extracellular signal-regulated kinase (Erk) in a Ras- dependent manner- resulting in cell scattering and inducing a morphological change known as "humming bird" phenotype in endothelial cells (Maroun *et al*., 2000, Neel *et al*., 2003). This phenotype is characterised by cell elongation and alteration of the actin cytoskeleton. Moreover, CagA-activated SHP2 has also been shown to dephosphorylate focal adhesion kinase (FAK) with subsequent elevation of cell motility due to reduction of active focal adhesions (Tsutsumi *et al*., 2006).

To prevent excessive CagA cytotoxic activity, a negative feedback loop is initiated upon translocation of the protein. Approximately 20% of phosphorylated CagA forms a complex by interacting with the carboxy-terminal Src kinase (Csk), which in turn inhibits the activity of SFK by tyrosine phosphorylation (Murakami *et al*., 2006). A similar inhibition effect has been shown for CagA that has not been SFK phosphorylated.

Numerous CagA interactions are known to occur without a need for phosphorylation of the protein. As previously mentioned, CagA localises to the host cell plasma membrane, where it can interact with the cell junction proteins zonola occludens-1 (ZO-1) and junction adhesion molecule (JAM) through recruitment of

SHP2, resulting in a weakened endothelial barrier and monolayer leakage (Amieva *et al*., 2003). CagA has also been shown to bind to growth factor receptor bound protein 2 (Grb2) and form a complex with son of sevenless (Sos) resulting in Ras activation and subsequent alterations in cell scattering and proliferation (Mimuro *et al*., 2002). In addition, CagA's ability to stimulate reactive oxygen species (ROS) production through mitochondria interactions elicits the same cellular effects (Handa *et al*., 2006). A morphological change to humming bird phenotype can be induced through CagA interacting with C-Met hepatocyte growth factor receptor (similar to activation of ERK) (Churin *et al*., 2003). Finally, recent data has proven CagA's ability to activate the nuclear factor of activated T cells (NFAT) in gastric epithelial cells- thus inhibiting progression of cell cycle (Yokoyama *et al*., 2005).

In summary, the cytotoxin-associated protein of *H. pylori* is responsible for many of the known pathogenic effects of the bacterium and can influence many aspects of cellular morphology and cycle. Studies of its secretion mechanism and subsequent pathway activations have revealed a diverse, multifunctional protein.

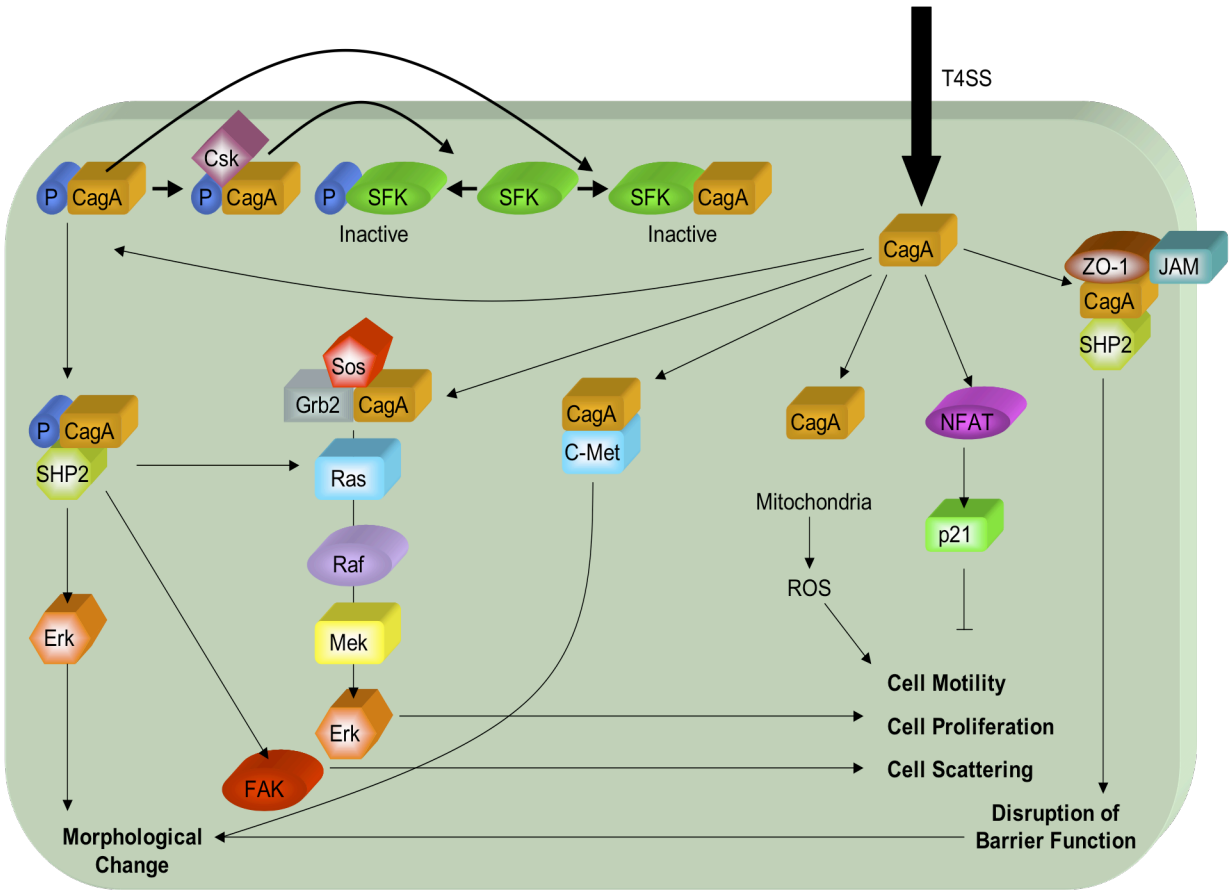


Fig. 1.4.6. CagA induced signal transduction. Translocation of CagA into the cell occurs through the Type IV secretion system. Its subsequent interactions can be tyrosine phosphorylation-dependent or -independent. Csk, C-terminal Src Kinase; SHP2, Src homology 2 (SH2) domain- containing tyrosine phosphatase; Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Grb2, growth factor receptor bound protein 2; JAM, junctional adhesion molecule; MEK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; ROS, reactive oxygen species; SFK, Src family kinase; Sos, son of sevenless; ZO-1, zonola occludens-1.

1.4.3.3 Urease

The enzyme urease accounts for approximately 10% of the total protein content of *H. pylori*, making it the bacterium's most abundant protein and virulent factor. As previously mentioned, VacA forms pores in cell membranes inducing the release of urea and anions from host cells. Urease converts this urea into ammonia and bicarbonate, aiding gastric colonization of the stomach by neutralizing the acidic conditions (Burne and Chen, 2000). Urease activity varies greatly between *H. pylori* strains despite being present in all (Contreras *et al*., 2003), and it has the ability to be reabsorbed by the bacterial cell surface through an autolytic mechanism (see section 1.4.3) or to exist in the bacterial cytoplasm (Phadnis *et al*., 1996). The ammonia produced has been shown to have cytotoxic effects on epithelial cells, thus promoting pathogenesis of the bacterium. In addition Fan *et al.* have reported a urease-dependent induction of apoptosis in gastric epithelial cells and it is known to activate monocytes and stimulate inflammatory cytokine production (Fan *et al*., 2000).

1.4.3.4 Heat Shock Proteins

All organisms respond to environmental stress with an upregulation of heat shock proteins (Hsp). The Hsp's are part of a group of approximately 24 proteins that are highly conserved across species and have been classified into 6 families based on molecular size: Hsp 100, 90, 70 60, 40 and small (Jolly and Morimoto, 2000). They function as chaperones to: facilitate the folding and unfolding of proteins and resolubilisation of protein aggregates, aid transmembrane molecular transport, and

are implicated in loading immunogenic peptides onto major histocompatibility complex for T-cell presentation (Schlesinger, 1990).

Characterisation of a 58-kDa protein secreted by *H. pylori*, which is also present on the bacterial surface, showed homology to Hsp's, and was hence named HspB- often referred to as *H. pylori* Hsp60 homologue (Dunn *et al*., 1992). HspB has been shown to have a role in adherence and attachment of *H. pylori* to the gastric epithelium and induce Interleukin-8 (Il-8) secretion, leading to gastric inflammation and increasing the risk of gastric carcinoma (Hayashi *et al*., 1998, Kawahara *et al*., 1999). The secretion of HspB (along with CagA, VacA and Urease positivity) is associated with a more virulent form of the bacterium. However, unlike the other *H. pylori* secreted factors, few studies have been carried out on HspB and little is known of its functions and host cell interactions.

1.4.3.5 Other Secretions

In culture *H. pylori* has also been shown to secrete the potent chemotactic factor Diethyl Phthalate, which has been shown to stimulate monocyte migration (Keire *et al.*, 2001).

1.4.3.6 Adhesions and Other Outer Membrane Proteins

While secreted proteins form the major virulence factors of *H. pylori* and contribute greatly to the pathogenesis of diseases associated with the bacterium, adhesion to host cells is necessary for prolonged gastric colonisation. Furthermore, CagA T4SS requires cell adherence to function, and many adhesions are themselves virulent factors- influencing inflammation and stimulating host immune responses. Complete genome sequencing of two strains (J99 and 26695) have led to identification of one major outer membrane family of 33 genes which encode the *H. pylori* outer membrane proteins (OMP) (Alm *et al.*, 1999, Tomb *et al*., 1997). Two sub-groups of this family have been identified- the Hop (*H. pylori* outer membrane protein) and Hop-related Hor families. Here we focus on adhesions and the Hop family, as little experimental data is available on Hor.

1) BabA (HopS): A 78 kDa protein encoded by the babA gene, it represents the best-characterised adhesion protein of *H. pylori*. It mediates binding to fucosylated Lewis b (Le^b) blood group antigens on host cells (Boren *et al*., 1993). BabA is suggested to have a role in pathogenesis of the bacteria as one of its alleles (babA2) is linked with peptic ulcer disease and gastric carcinoma (Gerhard *et al*., 1999).

2) OipA (HopH): A 34 kDa protein, which may function in adhesion as well as being a pro-inflammatory response-inducing protein (Yamaoka *et al.*, 2000). Conjointly, experimental data reveals OipA expression is concurrent with increased Il-8 expression *in vitro* and *in vivo* (Ando *et al.*, 2002).

3) SabA (HopP): During *H. pylori* gastric colonisation and inflammation, non-sialylated Lewis antigens are replaced with sialylated Lewis antigens. The role of SabA is to mediate binding to sialic acid-containing glycoconjugates (Mahdavi *et al*., 2002).

4) Lipopolysaccharide (LPS): LPS is a large molecule, and forms the major component of the gram-negative bacteria cell wall. It is comprised of three parts: polysaccharide (O) side chains, core oligosaccharide, and lipid A. It gives structural integrity to the bacteria and protects it from some chemicals. *H. pylori* LPS contains fucosylated oligosaccharide antigens that are similar to Lewis antigens structurally and immunologically. Thus, they are thought to contribute to immune evasion (Lozniewski *et al*., 2003). Unlike LPS of other bacteria, this molecular mimicry means only a weak innate immune response is stimulated.

5) *H. pylori*-neutrophil activating-protein (HP-NAP): HP-NAP is a 200-kDa highly symmetrical dodecameric molecule that belongs to the Dps (DNA-binding proteins from starved cells) protein family. These proteins are generally expressed by bacteria under environmental stress to protect DNA against oxidative damage. It adheres to gastric epithelium and, upon crossing the epithelial lining, recruits monocytes and neutrophils to the site of infection. Moreover, it has the ability to survive under oxidative stress conditions, play a role in the pathogenesis of *H. pylori* infection by acting as a major antigen and inducing an immune response, and acts as an adhesion for the bacteria (Evans *et al*., 1995, Montemurro *et al*., 2001, Montemurro *et al*., 2002).

6) AlpA and B: Inactivation of the AlpA and B genes reduce adherence of *H. pylori* to gastric epithelial cells.

7) Flagella: *H. pylori* has 4-6 flagella, which are essential for motility and colonization of the gastric epithelium. Similar to LPS, it does not induce a strong immune response, hence aiding evasion of the host immune system.

1.4.4 The Immune Response to *H. pylori*

The immune system is a collection of mechanisms within an organism that protects against infection by identifying and killing pathogens. Two immune systems operate in concert within the human body, the innate and acquired systems. Innate immunity refers to the antigen-nonspecific defense mechanisms that a host uses immediately or within a number of hours after exposure to almost any antigen (Janeway Charles; Paul Travers, Stvrtinova, 1995). Adaptive immunity on the other hand, refers to antigen-specific defense mechanisms that take several days to become protective and are designed to react and remove a specific antigen; this type of immunity continues to develop throughout life (Alberts, 2002, Janeway CA, 2005). The immune response to *H. pylori* involves both innate and acquired immunity, with various bacterial molecules inducing the response.

Innate immunity to bacterial molecules centers on Toll-like receptors (TLRs) expressed on antigen-presenting cells (APCs) such as monocytes and dendritic cells (DCs). Secretion of pro-inflammatory cytokines interleukin (IL)- 1β and IL-8, along with tumor necrosis factor-α (TNF-α) is induced following bacterial contact with APCs. In turn, these cytokines induce granulocytic infiltration in a chemotactic

manner (Crabtree, 1996b). In general, innate studies have focused on LPS of *H. pylori* and TLR-4, as the LPS of gram-negative bacteria elicit a strong immune response. However, as previously noted, the *H. pylori* LPS does not stimulate innate immunity due to its similarity to Lewis antigens. This fact was reflected in experimental data with gastric cells lines showing no response to the LPS (Backhed *et al*., 2003). As TLR2 and 5 are also known to respond to gram-negative LPS and flagella, studies were conducted to determine whether they could recognize and respond to *H. pylori*. Data revealed that epithelial cell cultures infected with the bacteria induced nuclear factor-κB (NF-κB) activity in cells transfected with TLR2 and 5, but not TLR4 (Smith *et al*., 2003). However conflicting reports show TLR5 evasion by *H. pylori* flagella in flagellin-responsive cell lines (Gewirtz *et al*., 2004). *In vitro* and *in vivo* studies consistently demonstrate that cell contact with *H. pylori* results in increased NF-κB and AP-1 expression (in a MAP Kinase- dependent manner), with a subsequent increase in IL-8 secretion (Aihara *et al*., 1997, Keates *et al*., 1997, Sharma *et al*., 1998).

The Adaptive immune cellular response is triggered primarily by attachment of *H. pylori* to epithelial cells and production of the antigenic HSPs, LPS and urease- all of which activate T-cells (following macrophage processing) (Di Tommaso *et al*., 1995). Attachment results in continuous gastric inflammation, with neutrophils, lymphocytes, plasma cells and macrophage all playing a role in attempting to rid the body of the bacteria. Mentioned previously was the effect of *H. pylori* on cellular junctions. This disruption enhances antigen presentation and greatly increases the immune response, resulting in increased IL-1, IL-6 IL-8 and TNF-α production

(Crabtree, 1996a, Fan *et al*., 1995). These cytokines are indicative of a Th1 response phenotype, and induction of interferon-γ (IFN-γ) and its related genes supports this (Tummala *et al*., 2004). Notably, host genetics also plays an important role in cellular adaptive immunity as the IL-1B gene encodes IL-1β- a major contributor to amplifying the *H. pylori*-associated inflammation (Noach *et al*., 1994).

Despite a strong systemic and local antibody release, binding of IgG to *H. pylori*, thereby stimulating phagocytosis and bacterial susceptibility to the process of complement, the humoral adaptive immune response is highly ineffective. In actuality, the response may aid pathogenesis of *H. pylori*, as shown by Israel and Peek *et al*., when delivery of the antibodies directed against the bacteria into mice induced gastritis (Israel and Peek, 2001). The failure of this system is largely put down to the gastric mucus in which *H. pylori* resides, as it provides a protective layer making the bacteria inaccessible to specific antibodies.

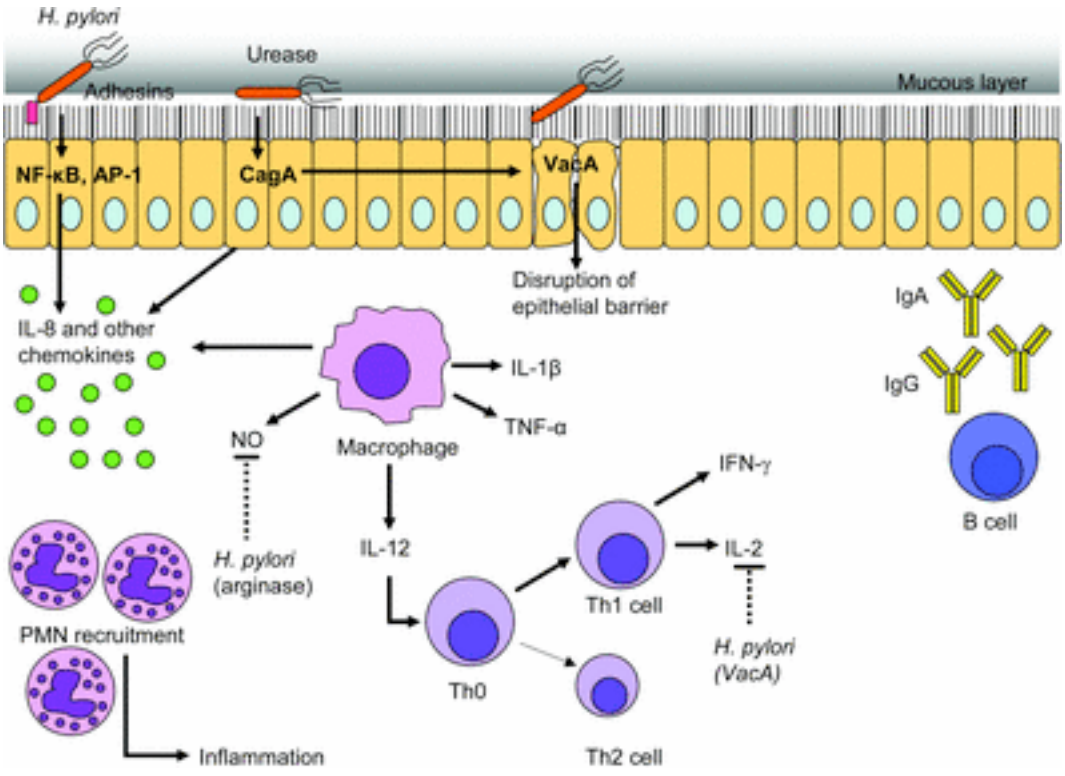


Fig. 1.4.7 The immune response to *H. pylori*. (Portal-Celhay and Perez-Perez, 2006)

1.4.5 Diagnosis, Treatments and Vaccines

Diagnosis of *H. pylori* has improved vastly in recent years and many non-invasive tests are now available. However, a combination of both invasive and non-invasive methods is still the gold standard for accurate identification of the bacteria (Dzierzanowska-Fangrat *et al*., 2006, Ricci *et al*., 2007, Vaira and Vakil, 2001). Each of the tests are outlined below:

Non –Invasive Methods

1) Urea breath test: This is a rapid diagnostic test. Patients swallow urea labeled with an uncommon isotope- either radioactive carbon-14 or non-radioactive carbon

13. Ten to thirteen minutes later, detection of isotope- labeled carbon dioxide in exhaled breath indicates that the urea was split through the action of *H. pylori* urease, and thus presence of the bacteria is confirmed (Surveyor *et al.*, 1989).

2) Stool Antigen Test: This test utilizes polyclonal anti-*H. pylori* antibody adsorbed to microwells. Diluted patient samples and a peroxidase-conjugated polyclonal antibody are added to the wells and incubated for one hour at room temperature. A wash is performed to remove unbound material. Substrate is added and incubated for ten minutes at room temperature. Colour develops in the presence of bound enzyme. A stop solution is added and the results are interpreted visually or spectrophotometrically. Any laboratory can perform the test in less than 90 minutes, since no special equipment is needed (Koletzko, 2005).

3) **Antibody testing:** This is a similar principle to the stool antigen test, anti-*H. pylori* antibodies are used in an ELISA and patient's blood is added to the wells to detect bacterial presence.

Invasive Methods

1) Endoscopy: with subsequent biopsy. Testing of biopic samples can occur in numerous ways: (a) Slide with a colour change indicator if urea is broken down- indicating a positive reaction for *H. pylori*. (b) Performing nested PCR with primers specific to conserved sequences only found in *H. pylori* strains. (c) Culturing the sample and testing using antibodies in a Western blot or immunocytochemical format (Ricci *et al.*, 2007).

Invasive methods have the advantage of a definitive positive or negative result, and estimation of bacterial load. However the nature of endoscopy is a drawback due to its discomfort. While non-invasive methods are quick, generally in-expensive and cause minimal discomfort, results can be hampered by factors such as smoking and alcohol, or other pharmaceuticals, which alter the pH of the stomach briefly and decrease the number of bacteria present. This can lead to incorrect estimation of bacterial load or even a negative test result.

The recommended *H. pylori* eradication therapy continues to be triple therapy with a proton pump inhibitor (PPI) and two antibiotics of clarithromycin, amoxicillin or metronidazole for seven days (Della Monica *et al*., 2002). PPI's inhibit gastric acid secretion by blocking the hydrogen/potassium adenosine triphosphatase enzyme system (proton pump) of the gastric cell. Clarithromycin prevents bacterial protein synthesis, thus inhibiting growth. Amoxicillin inhibits the synthesis of bacterial cell wall and metronidazole selectively disrupts nucleic acid synthesis in anaerobic bacteria.

However, in 20% of cases this therapy fails and hence the requirement exists for continued revision and updating of treatments. For this reason the top six treatment regimes have been identified. Failure of one to eradicate *H. pylori* means moving down the list to the next best and rigorous treatment.

1) Clarithromycin, amoxicillin, and PPIs for seven days. (This is the 1st line defense, and works in 80% of cases).

2) Longer duration of therapy.

3) A Quadruple therapy- PPI, Bismuth, tetracycline and metromidazole.

4) Sequential treatment: Five day PPI, amoxicillin, followed by five day PPI, clarithromycin and metromidazole.

5) Adjuvant therapy

6) New anti-microbial-based therapies: Seven day PPI triple regime with levofloxacin and amoxicillin.

As with most diseases, prevention is preferential to curing an established infection. For this reason, the development of a vaccine against *H. pylori* is underway and while none are commercially viable at the moment, clinical trials suggest it is only a matter of time. Immunization with *H. pylori* urease subunit in conjunction with the mucosal adjuvants cholera toxin (CT) or heat labile toxin of *Escherichia coli*, into oral (Michetti *et al*., 1994, Weltzin *et al*., 1997), nasal (Weltzin *et al*., 1997), or rectal (Kleanthous *et al*., 1998) passageways, can impart protection against *H. pylori* infection in mice and ferrets (Cuenca *et al*., 1996). Moreover, due to gastric mucosal colonization by the bacteria, mucosal vaccinations have also been explored in humans. Unfortunately these studies revealed weak urease immunogenicity, although the safety of recombinant urease was confirmed (Kreiss *et al*., 1996, Michetti *et al*., 1999, Banerjee *et al*., 2002). The main conclusion taken from these studies was that it is necessary to increase our knowledge on the mechanisms by which *H. pylori* is cleared following vaccination, hence creating a better

understanding of the host defense systems involvement, leading to improvements in future vaccine design.

1.5 *H. pylori* and Atherosclerosis

Recently, a considerable number of studies have focused on whether any correlation exists between *H. pylori* infection and the complex process of atherosclerosis. To this end, research has concentrated on whether bacterial positivity affects known atherosclerotic lesion markers such as plasma levels of cytokines, fibrinogen, lipids and C-reactive protein (CRP)- a marker of inflammation. Much controversy surrounds this field with many studies reporting findings for, or against, the association. These data are presented below.

Higher levels of all these lesion markers were shown in patients with coronary heart disease (CHD) and *H. pylori* infection than in controls (Pieniazek *et al*., 1999). Zito *et al.* (1999) reported elevated fibrinogen levels and Hoffmeister *et al.* (2001) showed decreased HDL cholesterol, both in *H. pylori* positive patients. Similar positive associations were shown by Majka *et al.* (2002) when examining plasma levels of cholesterol, LDL-cholesterol, fibrinogen and IL-8. In addition, separate studies reveal seropositivity to *H. pylori* correlates with higher levels of tumour necrosis factor alpha (TNFα) and fibrinogen in patients with coronary artery disease (CAD) (Schumacher *et al*., 2002). A polymerase chain reaction and immunohistochemical examination of 38 atherosclerotic plaques by Ameriso *et al.* (2001) found *H. pylori* DNA present in 20 plaques, with 10 of these testing positive for immunohistochemical staining. Similarly, Kowalski *et al.* (2002) provided

evidence of *H. pylori*-related DNA in 47.8% of patients with CAD. Previously mentioned was the role of adhesion molecules in the initiation and pathogenesis of atherosclerosis. Interestingly, soluble ICAM-1 and VCAM-1 levels have been found to be associated with IgA seropositivity to *H. pylori* (Maciorkowska *et al*., 2005). Moreover, mean anti- Hsp65 titers (Hsp is secreted by *H. pylori*), which correlate to the severity and extent of coronary atherosclerosis, decreased in 100 patients following eradication of the bacteria (Birnie *et al*., 1998). Finally, many studies have addressed the role of CagA- positive *H. pylori* strains (associated with a more virulent infection) in cardiovascular disease, with Pasceri *et al*. (2008), Franceschi *et al*. (2002) and Mayr *et al*. (2003) all confirming an association of CagA with atherosclerosis, and significantly in the case of Mayr *et al*. increased expression of C-reactive protein was found in individuals infected with CagA- positive strains versus CagA- negative.

An interesting argument is the manner in which *H. pylori* may interact with endothelial cells. Immune function would dictate that if the whole bacterium were to vacate the stomach through an ulcer or other means, it would immediately be destroyed. This has lead to suggestions that the bacterium influences progression of the disease through the damaging influence of its products (i.e. cytokines, endotoxins, cytotoxins and other virulence factors), with many of the above studies supporting this hypothesis.

Much evidence exists against the association between *H. pylori* and atherosclerosis. Ridker *et al*., Wald *et al*. and Biagi *et al*. all reported no association between *H. pylori* seropositivity and myocardial infarction (MI) (Ridker *et al*.,

2001), IHD (Wald *et al*., 1997) and CHD respectively (Biagi *et al*., 2000). In addition, numerous studies have rejected the links between increased atherosclerotic markers such as CRP, fibrinogen, oxidized LDL, leukocyte counts and pro-thrombin in response to *H. pylori* infection (Brenner *et al*., 1999, Hoffmeister *et al*., 2001, Koenig *et al*., 1999, Parente *et al*., 2000, Rothenbacher *et al*., 2001, Schweeger *et al*., 2000, Singh *et al*., 2002, Whincup *et al*., 2000, Yusuf and Mishra, 2002). Lastly, research focusing on the role of CagA- positive *H. pylori* strains in IHD, and detection of bacterial DNA in atherosclerotic plaques found no such links (Danesh *et al*., 1999, Danesh and Peto, 1998, Danesh *et al*., 2000, Koenig *et al*., 1999, Pellicano *et al*., 2002, Whincup *et al*., 2000).

In order to clarify the putative role of *H. pylori* in vascular disease and in particular atherosclerosis, it is necessary to examine the effect of the bacteria on the central mediator of lesion formations- the endothelium. Several *in vitro* studies describe profound effects of *H. pylori*-derived components on vascular endothelial properties and function. Incubation of microvascular endothelial cells with *H. pylori* extracts, or co-culture with the bacterium, has been shown to decrease cell viability (Kalia *et al*., 2001) and inhibit angiogenesis (Kalia *et al*., 2001, Jenkinson *et al*., 2002) A recent study by Pearce *et al*. (2004) has shown that *H. pylori* extracts can inhibit proliferation in both human dermal microvascular endothelial cells (HDMvECs) and human umbilical vein endothelial cells (HUVECs). Moreover, Kurosawa *et al*. also demonstrate that *H. pylori* extracts inhibit proliferation, whilst inducing apoptosis in HUVECs, in a CagA/VacA-independent manner (Kurosawa *et al*., 2002). Other recent studies have also shown that *H. pylori* co-culture and *H.*

pylori-conditioned medium significantly increase recruitment and transendothelial migration of neutrophils (Brisslert *et al*., 2005, Innocenti *et al*., 2002).

In summary, cardiovascular disease refers to dysfunction of the heart and blood vessels and accounts for approximately one third of global deaths annually. Maintenance of a healthy endothelium is central in preventing the onset of cardiovascular disease and its development to a pathologic condition. Regions of the vasculature where blood-flow is perturbed, such as arterial bifurcations or vessel curvature, are preferential initiation sites for occlusive pathologies. At these locations, owing to reduced endothelial function and the presence of cardiovascular disease risk factors an inflammatory, pro-atherogenic phenotype predominates. However, traditional risk factors can only account for approximately 50% of atherosclerotic cases, and thus, research has focused on identifying other factors involved in the disease. Following discovery of its DNA in atherosclerotic plaques, the Gram-negative bacterium *Helicobacter pylori*, has been proposed as one such non-traditional risk factor. Numerous studies point to a positive association between *H. pylori* infection and markers of atherosclerosis such as C-reactive protein and adhesion molecule expression. Moreover, toxins secreted by the bacteria have known effects in promoting immune and inflammatory responses and importantly, disrupting endothelial function.

Whilst studies have examined the putative role of *H. pylori* in this respect, cell models employed thus far are quite diverse, and provide little or no information on cells of aortic or coronary artery origin where atherosclerotic plaque formation predominates. To address this knowledge deficit, the effects of *H. pylori* 60190 (a

strain positive for VacA, CagA and Urease) on apoptosis, proliferation, tube formation, migration, and tight junction integrity, was investigated, all in a single aortic endothelial cell model (BAEC). Moreover, as previously noted by the author, the major secreted virulence factor of the bacterium in culture is the VacA cytotoxin. To this end, the role of VacA on endothelial cell nitrite production- an early indicator of endothelial dysfunction was examined.

Chapter 2

Materials and Methods

2.0 Material & Methods:

All reagents used in this study were of the highest purity commercially available and were of cell culture standard when applicable.

2.1 Materials:

The American Type Culture Association (ATCC), (Middlessex, England)

Helicobacter pylori Strain 60190 (ATCC 49503)

AGB Scientific (Dublin, Ireland)

Syringe Filters

96-well white plates

Amersham Pharmacia Biotech (Buckinghamshire, UK)

Anti-rabbit 2^0antibody, HRP-conjugated

BIOMOL Gmbh (Hamburg, Germany)

E-coli BL21 strain

Coriell Cell Repository (Camden, New Jersey)

Bovine Aortic Endothelial Cells

DakoCytomation (Glostrup, Denmark)

Polyclonal Rabbit Anti-Human Von Willebrand Factor

Fluorescent Mounting Media

Fisher Scientific (Leicestershire, UK)

Buffer Solution pH 4 (phthalate)

Buffer Solution pH 7 (phosphate)

Buffer Solution pH 10 (borate)

Millipore (Cork, Ireland)

6-well Millicell® Hanging Cell Culture Insert

Molecular Probes (Oregon, USA)

Alexa Fluor® 488 F (ab')$_2$ fragment of goat anti-mouse IgG (H+L)

Alexa Fluor® 488 F(ab')$_2$ fragment of goat anti-rabbit IgG (H+L)

Alexa Fluor 546 Phalloidin

Vybrant® Apoptosis Assay Kit

Vybrant® CFDA-SE Cell Tracer Kit

Nalgene (Rochester, New York)

Cryogenic Vials

Cryo Freezing Container

Pierce Chemicals (Cheshire, UK)

BCA Protein Assay Kit

Roche (Dublin, Ireland)

Complete Protease Inhibitor

Sarstedt (Wexford, Ireland)

1.5 mL micro tube with safety cap

10, 200 and 1000 μL pipette tips

15 and 50 mL falcon tubes

25 mL sterilin tubes

5, 10 and 25 mL serological pipettes

6-well tissue culture plates

T-175 tissue culture flasks

T-25 tissue culture flasks

T-75 tissue culture flasks

Sigma Chemical Company (Poole, Dorset, England)

2-propanol

2,3 Diaminonapthalene

Ac-DEVD-pNA

AEBSF

Aproprotinin

Ammonium Persulfate

Agarose

Acridine orange/Ethidium Bromide dual stain

Bovine Serum Albumin

Brightline Haemocytometer

Bromophenol Blue

Chloroform

Collagen, Type 1 Rat Tail

Fetal Bovine Serum

FITC-Dextran (FD-40)

Formaldehyde

Glycine

Hanks Balanced Salt Solution

HEPES

Hydrochloric Acid

Lauryl Sulfate (i.e. Sodium Doecyl Sulphate (SDS))

Leupeptin

Mitomycin C

Penicillin-Streptomycin (100x)

Phosphate Buffered Saline

pNitroanilide

Ponceau S Solution

Protease Inhibitor Cocktail

Potassium Chloride

Potassium Phosphate

Potassium Phosphate-Dibasic Trihydrate

Potassium Hydroxide

RPMI 1640

Sodium Chloride

Sodium Deoxycholate

Sodium Nitrite

Sodium Orthovanadate

Sodium Phosphate-Dibasic (anhydrous)

Sodium Phosphate-Monobasic (anhydrous)

S-nitroso-*N*-acetylpenicillamine

Triton® X-100

Trizma Base

Trypan Blue

Trypsin-EDTA (10x)

Tween® 20

Zymed Laboratories (CA, USA)

Mouse anti-ZO-1 Monoclonal Antibody

2.2 Cell Culture Methods

All cell culture procedures were carried out under clean, sterile conditions using a Bio Air 2000 MAC laminar flow unit. Cells were monitored daily using and Olympus CK30 phase contrast microscope.

2.2.1 Culture of Bovine Aortic Endothelial Cells (BAECs)

Cryopreserved passage four Bovine Aortic Endothelial Cells (BAEC) were obtained from Coriell Cell Repositories, (catalogue no. AG08500) New Jersey, USA. Cells were routinely grown in RPMI-1640 media, supplemented with 10% (v/v) foetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL Streptomycin to give RPMI-complete media. Cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C and cultured in T25 cm^2, T75 cm^2 and T175 cm^2 flasks and 6 well plates. All experiments were carried out on cells between passage 5 – 15.

2.2.2 Trypsinization of BAECs

As BAECs are an adherent cell line, trypsinization was required for their sub-culture. Briefly, growth media was removed by aspiration and the cells washed in Hank's balanced salt solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FCS. An appropriate volume of trypsin/ethylenediamene tetracetic acid (10% v/v Trp/EDTA in HBSS) was subsequently added to the cells

and incubated for 1 – 2 min at 37^0C, until the cells were rounded, but not fully detached, and then tapped briefly to detach them from the growth surface. Growth media containing FCS was added to prevent further trypsinization and cells removed from suspension by centrifugation at 1,000 g for 5 min at 4^0C. Cells were resuspended in growth media (or freeze media) and either counted using a bright line haemocytometer for experiments, split at a 1:5 ratio for further subculturing or cryopreserved.

2.2.3 Cryogenic Preservation and Recovery of Cells

For long-term storage of cells, BAECs were stored in a commercial liquid nitrogen cryofreezer unit. Following trypsinization, cells were centrifuged at 1,000 x g for 5 min at 4^0C and supernatant was removed. The resultant pellet was resuspended in an appropriate volume of freezing media (RPMI-1640 media containing 20% v/v FCS, 10% DMSO- Dimethyl sulfoxide, and antibiotics), transferred as 1 mL aliquots to Nalgene cryogenic vials and frozen in a -80^0C freezer at a rate of -1^0C/min in a Nalgene cryo-freezing container. Cryovials were stored in the cryofreeze unit until required. For recovery of cells, cryovials were heated rapidly in a 37^0C water bath and added to a T 75cm^2 flask containing 15 mL of growth media to dilute the DMSO. After 24 h (approximately 80-90% plating efficiency), the media was removed and the cells were washed in HBSS and fresh growth media added.

2.2.4 Cell Counts

Following trypsinization, cell counts were performed using a brightline haemocytometer in conjunction with Trypan blue staining to assess cell viability. 20 μL of trypan blue was added to 100 μL of cell suspension and incubated at room temperature for 2 min. 10 μL of this suspension was added to the counting chamber of the haemocytometer (Brightline, Sigma) and visualized under phase contrast microscopy. Dead cells stained blue, whilst viable cells excluded the dye and appeared colourless. The number of viable cells was calculated according to the equation:

Avg. cell no. x 1.2 (dilution factor) x 1x10^4 (area under coverslip) = Viable cells/mL

2.2.5 Bacterial Growth and Fraction Generation

H. pylori 60190 (ATCC 49503- supplied as dessicate) was cultured on Pylori Agar Plates (BioMérieux®, Marcy l'Etoile, France) at 37ºC under microaerophillic conditions (using Oxoid gas generating kit) and incubated for 7 days to allow sufficient growth to occur. For **conditioned media preparation**; (Forsyth and Cover, 2000) bacterial colonies were scraped off and re-suspended in 3 mL phosphate buffered saline (PBS). The optical density (OD) of the suspension was determined at 600 nm and adjusted to a final OD of 1.0 in PBS, corresponding to 5X10^9 *H. pylori* bacteria/mL. (Innocenti *et al.*, 2002). Exactly 2.5 ml of bacterial suspension was added to a T25 tissue culture flask containing 40 mls of RPMI

complete media and incubated at 37^{o}C under microaerophillic (low oxygen) conditions for 24 h. Following this, conditioned media was sterile filtered using a 0.2 μM filter to yield *H.pylori*-conditioned media (*HPCM*). Dilutions of *HPCM* in RPMI complete media were routinely prepared for individual experiments (i.e. undiluted = 100%, 1:2 dilution = 50% etc.). *E-coli* and 60190:VacA negative (kindly gifted by Prof. John Atherton, Nottingham University) conditioned media was prepared in a similar manner. Experiments typically involved exposure of BAECs to either 1 ml/well (i.e. migration, tube formation, 24-well format) or 2 ml/well (i.e. apoptosis, proliferation, immunocytochemistry, 6-well format) of control or test media. The chloride channel inhibitor NPPB (100 μM) benzoic acid (Gojova and Barakat, 2005), Urease (10 μg/mL) (Enarsson *et al.*, 2005), *S*-nitroso-*N*-acetylpenicillamine (SNAP) and L-NAME (nitrite inhibitor) were included in *HPCM* when required.

Outer membrane protein extraction was carried out according to the method of Utt *et al.* (Utt *et al.*, 2002). Bacterial colonies were harvested by applying 1.5 mLs of sterile PBS pH 7.2 to each plate and gently scraping the cells from the plate surface. Subsequently, cells were washed once using PBS and re-suspended in 0.2 M glycine hydrochloride (pH 2.2) at a concentration of 4 g cells/100 mL. This suspension was then stirred at 20°C for 15 min and supplemented with complete protease inhibitors (Roche, Dublin, Ireland). Cells and cell debris were removed by centrifugation at 12000 x g for 15 min at 8°C. NaOH was added to the resultant supernatant to neutralise the acidity generated by the glycine-hydrochloride. This solution was dialysed against PBS for 18 h at 4°C to yield a concentrated OMP

fraction. Following BCA assay for protein concentration, appropriate concentrations of OMP were added to RPMI complete media (typical additions were 0-8 μg/mL). *E-coli* outer membrane proteins were prepared in a similar manner. Both *HPCM* and outer membrane protein fractions were routinely stored at -80°C and thawed as required.

2.2.6 Laminar Shear Stress Studies

For laminar shear stress studies, BAECs were seeded at 1 x 10^4 cells/cm^2 in 6 well plates and allowed to come to confluency, typically 2 – 3 days. Following this, media was removed and replaced with 4 mL of fresh growth media. Cells were then exposed to 0-10 dyne/cm^2 of shear stress for 24 h on an orbital shaker (Stuart Scientific Mini Orbital Shaker OS) set to the appropriate RPM as determined by the following equation (Hendrickson *et al*., 1999).

$$ShearStress = \alpha\sqrt{\rho n(2\pi f)^3}$$

Where α = radius of rotation (cm)

ρ = density of liquid (g/L)

n = 7.5 x 10^{-3} (dyne/cm^2 at 37^0C)

f = rotation per second

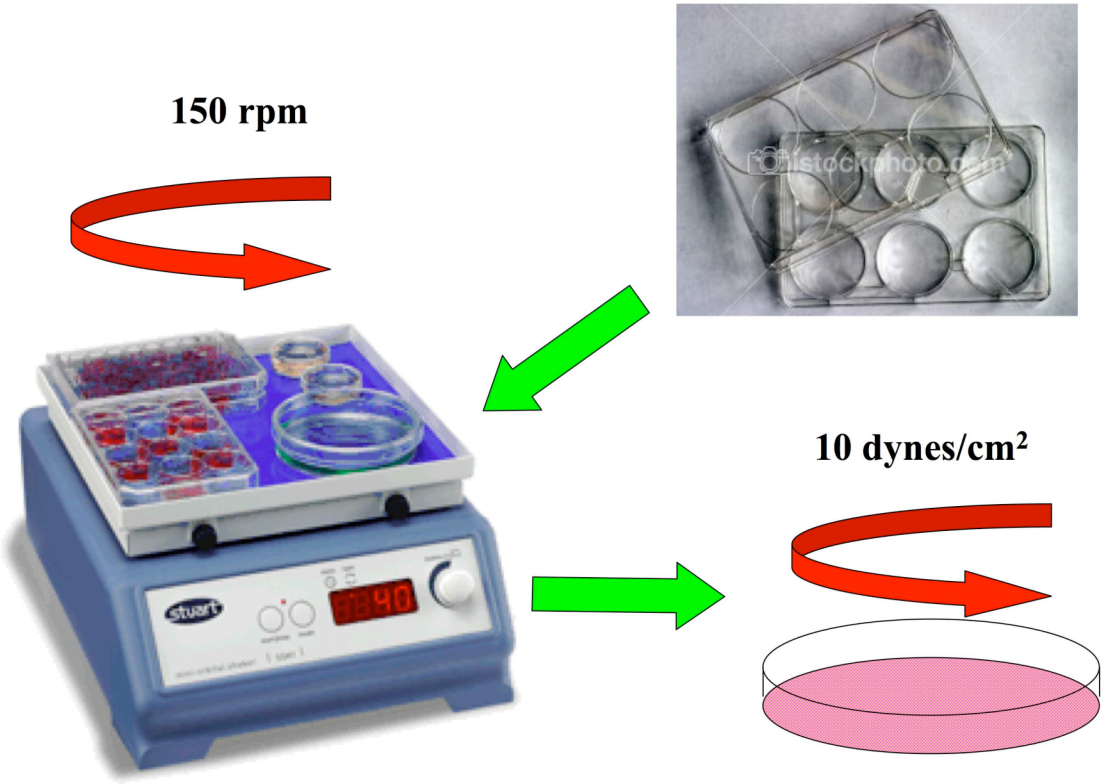


Figure 2.2.1: Apparatus used in non-pulsatile laminar shear stress studies.

2.2.7 Cyclic Strain Using the Flexercell® System

2.2.8

The Flexercell Strain Unit (Dunn Labortechnik, Germany) is an innovative microprocessor-driven instrument that regulates pressure to flexible bottomed Pronectin-coated BioFlex® plates to mechanically challenge cells in culture. It allows vascular cells to be exposed to a defined physiological level of cyclic strain *in vitro*. These two unique, novel, complementary technologies allow vascular endothelial cells to be examined under conditions of physiological pulse pressure

and cyclic strain and therefore scientific findings will realistically reflect the behaviour of the vasculature in response to haemodynamic forces.

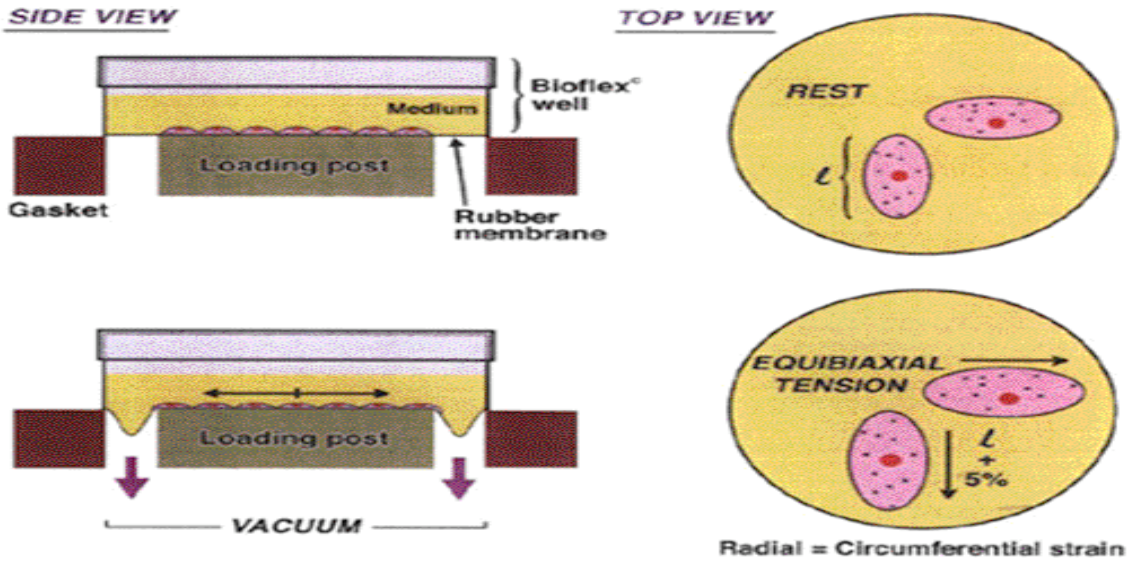
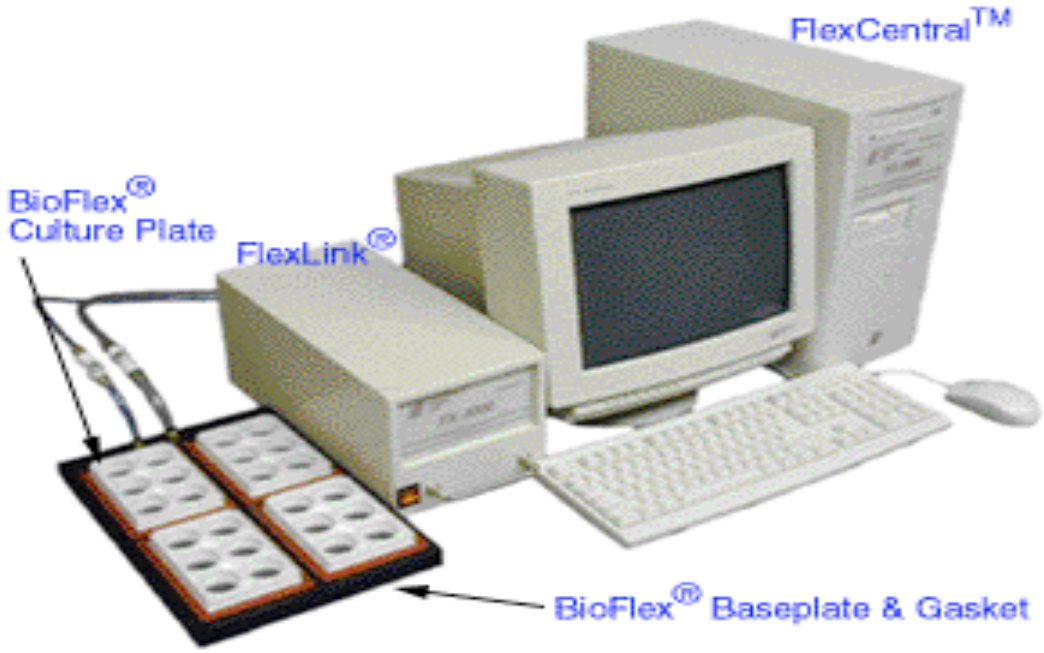


Fig 2.2.2a (above)- Effect of the Flex system on cells

Fig 2.2.2b (below)- The Flexercell® System



Flexercell® Procedure

BAECs were trypsinised and counted as described earlier**.** Each BioFlex® well is seeded with 1 x 10^5 cells and left to grow to confluency for 48 hours. After the cells have come to approximately 60-70% confluency the BioFlex® plate is mounted into the Flexercell® system, by gluing the loading post to the flexible membrane. The computer program corresponding to 10% strain is initiated and left running for exactly 24 hours, during this period the cells are kept incubated at 37°C with 5% CO_2 with humidity. A second BioFlex® plate, seeded as a control, remains unflexed (thus exposing it to 0% cyclic strain). Once the time course has finished the BioFlex® plate is removed from the baseplate and gasket, with subsequent excision of BioFlex® membranes for immunocytochemical staining.

2.2.7 Immunocytochemistry

In order to visually monitor the expression and/or subcellular localization of proteins, cells were prepared for immunocytochemical analysis as previously described (Groarke *et al*., 2001) with minor modifications. Cells were washed twice in phosphate buffered saline (PBS) and fixed with 3% (v/v) formaldehyde for 15 min. Cells were subsequently washed, permeabilised for 15 min with 0.2% (v/v) Triton X-100 and blocked for 30 min in 5% (w/v) BSA solution. Following

blocking, cells were incubated with the appropriate primary anti-serum or stain as indicated in table 2.2.7.1, followed by 1 h incubation with 1:400 dilution of either Alexa Fluor 488-conjugated anti-mouse or anti-rabbit secondary anti-serum. Nuclear DAPI staining was routinely performed by incubating cells with 0.5 x 10^{-6} μg/mL DAPI for 3 min. Cells were sealed with coverslips using DAKO mounting media (DAKO Cytomation, Cambridgeshire, UK) and visualized by standard fluorescent microscopy (Olympus BX50).

Primary Antiserum/Stain	Concentration/Dilution	Time (h)
Rhodamine Phallodin (actin stain)	1:200	1
anti-Von-Willebrand (endothelial marker)	1:1000	3
anti-ZO-1 (tight junction marker)	0.25 μg/mL	2

Table 2.2.1: Primary anti-sera and protein stains.

2.2.8 Permeability Studies

To measure barrier function in BAECs, a permeability assay was employed. Following laminar shear stress studies in 6-well plates, cells were trypsinized and replated into Millipore®-Clear Inserts at 2.5 x 10^5 cells/cm^2. After 24 h when cells were confluent, transendothelial permeability was measured as previously described (Collins *et al*., 2006). At t=0, 40 kDa fluorescein isothiocyanate (FITC)-labelled

dextran was added to the abluminal chamber (to give a final concentration of 250 μg/mL) and diffusion of dextran across the monolayer allowed to proceed at 37°C for 2 h. Media samples (30 μL) were collected every 30 min from the subluminal compartment and monitored in triplicate (7 μL sample + 93 μL media) for FITC-dextran fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively (Perkin-Elmer Luminescence Spectrometer LS50B with microplate reader attachment). RPMI-complete media without FD40 dextran was used as a blank. % Trans Endothelial Exchange (%TEE) of FITC-dextran 40 kDa is expressed as the total subluminal fluorescence at a given time point (from 0-120 min) expressed as a percentage of total abluminal fluorescence at t=0 min.

2.2.9 Preparation of Whole Cell Lysates

Post- treatment confluent BAEC monolayers in 6-well dishes were washed three times with HBSS and solubilised by the addition of 0.25 mL/well lysis buffer and incubation at 4°C for 1 h with agitation. Lysis buffer was a modified radioimmunoprecipitation assay (RIPA) buffer (49.92 mM HEPES- pH 7.5, 149.76 mM NaCl, 1% v/v Triton X-100, 0.5% w/v sodium deoxycholate and 0.1% w/v SDS) supplemented with 0.1 M NaF, 5 mM EDTA- pH 8, 0.01 M $NaPO_4$, 1.04 mM AEBSF, 0.08 μM aprotinin, 0.02 mM leupeptin, 0.04 mM bestatin, 0.015 mM pepstatinA, 0.014 mM E-64. Insoluble material was pelleted by centrifugation for 15 min at 12,000 x g and the supernatant removed to fresh microfuge tubes. BCA

protein assay was performed, following which samples were stored at -80ºC until analysis was carried out.

2.2.10 Caspase-3 Assay

Caspase-3 activity, an index of apoptosis, was monitored according to the method of Sweeney *et al*. (Sweeney *et al*., 2004). 10 µl of Caspase-3 substrate (2 mM Ac-DEVD-pNA containing 10% DMSO in 20 mM HEPES, 0.1% CHAPSO, 5 mM DTT and 2 mM EDTA) was added to 10 µl cell lysates and diluted in assay buffer (20 mM HEPES, 0.1% CHAPSO, 5 mM DTT and 2 mM EDTA) to a final volume of 100µl. Samples were incubated for 90 min and the absorbance measured at 405nm using a Biotek® ELx800 plate reader. Appropriate negative controls and blanks were included in the assay. A pNitroanilide (pNA) standard curve (0-200 µg/µl) was used for calculation of Caspase-3 specific activity worked out in µg substrate cleaved/ min/ µg protein at 37°C and converted to fold change relative to control for data presentation.

2.2.11 Acridine Orange/Ethidium Bromide Cell Staining

The staining of BAEC's with the Acridine Orange/Ethidium Bromide (AO/EtBr) dual stain (10 µg/mL) allows concurrent determination of viable, apoptotic and necrotic cells. Procedures were carried out as per Sweeney *et al*.

(Sweeney *et al.,* 2004). Post- treatment, BAECs were washed twice in PBS and fixed in ice cold isopropanol for 5 min. Cells were then rehydrated in 1x PBS for 8 min, and stained with the AO/EtBr dual nuclear stain for 5 min, rinsed with PBS and visualized by standard fluorescent microscopy (Olympus BX50).

2.2.12 FACS Analysis

Post treatment, both apoptotic and proliferative BAEC phenotypes were monitored using flow cytometery as described by Gao *et al.*, (Gao et al., 2007). **Apoptosis:** BAEC monolayers were washed once in HBSS and harvested by trypsinisation. Cells were pelleted by centrifugation at 1000 x g for 5 min and the supernatant removed. Cell pellets were washed by the addition of 1 mL ice-cold 1X PBS containing 0.1% BSA and resuspended by gentle pipeting. This was followed by centrifugation at 1000 x g for 5 min. Supernatant was discarded and the cells resuspended in 100 µl of 1X Annexin-Binding Buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCL_2$, pH 7.4). Propidium Iodide (0.4 µl from 100 µg/mL working solution) and AlexaFluor 488 Annexin V (1 µl of 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4 plus 0.1% BSA) were added to the cell suspension and incubated at room temperature for 15 min. Following incubation, a further 100 µl of 1X Annexin-Binding buffer was added to the cells with gentle mixing.

Proliferation: $2X10^4$ BAEC's were seeded into 6-well plates and allowed to grow for 24 h. Subsequently the growth media was removed and cells were washed once with HBSS. A working solution of 5 µM CFDA SE (carboxy-fluorescein

diacetate succinimidyl ester) was prepared in an appropriate volume of HBSS to allow the addition of 1 mL CFDA-HBSS per well. CFDA-HBSS was added to the wells and cells incubated for 15 min at 37°C. Following incubation, the CFDA-HBSS was removed and replaced with fresh, pre-warmed media and cells were allowed to recover for 12 h before overnight quiescence. Cells were treated with *HPCM* (0-25% dilutions) or OMP and harvested every 24 h for 5 days, pelleted by centrifugation at 1000 x g for 5 min and the supernatant removed. Cells were washed with 1 mL ice-cold 1X PBS containing 0.1% BSA and resuspended by gentle pipeting. This was followed by centrifugation at 1000 x g for 5 min before being resuspended in 100 μl of ice-cold 1X PBS containing 0.1% BSA.

Both apoptotic and proliferative samples were then placed on ice and analysed using a Becton Dickinson FACSCAN flow cytometer.

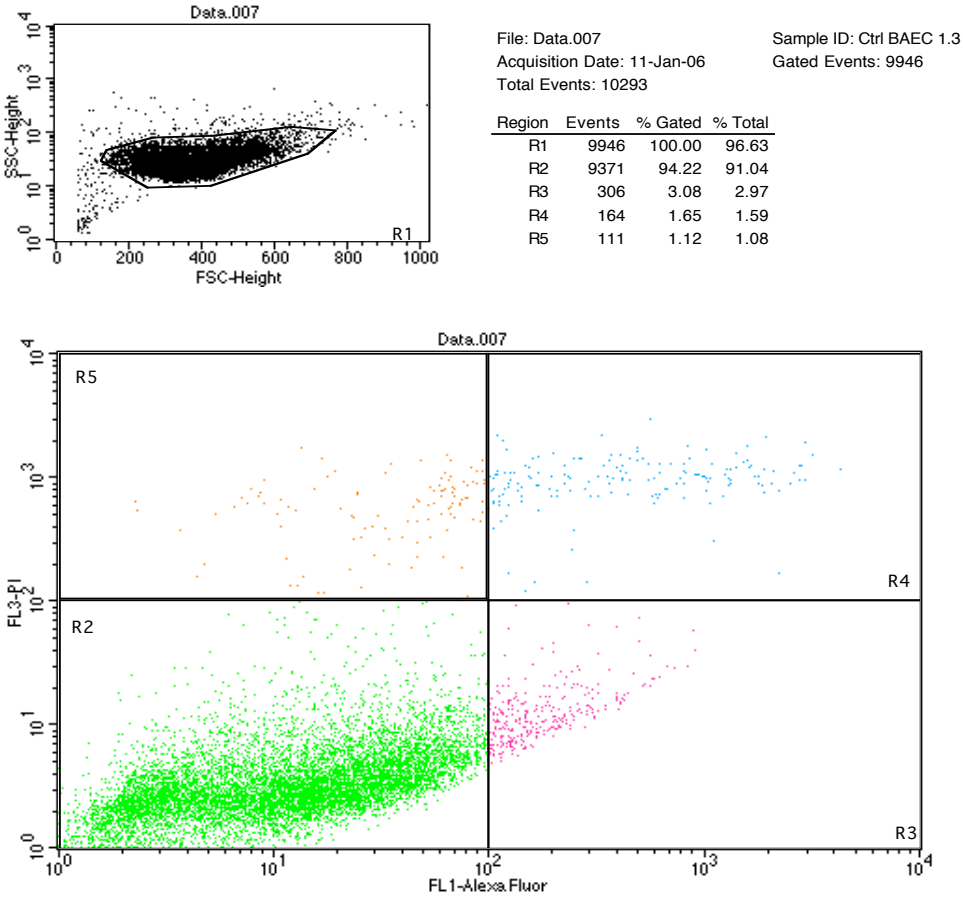


Fig. 2.2.3 Typical FACS analysis for apoptosis. R2= healthy cells (green), R3= Early apoptotic cells (purple), R4= Late apoptotic cells (blue), R5= Necrotic cells (orange). Apoptotic values for healthy BAEC ranged from approximately 1-4%.

2.2.13 Wound Heal Assay

Wound heal assay was carried out as described by de Jonge *et al* (de Jonge *et al*., 2002). BAEC's were seeded at $5x10^4$ cells/well of a 24-well plate and allowed to grow for 24 h, following which they were quiesced overnight and treated with

HPCM or OMP containing mitomycin C (2.5 µg/mL). *In vitro* wounds were created by scraping BAEC monolayers with a yellow tip. After injury, the wound was photographed at 2 distinct positions every 2 h and the distance between the two wound edges calculated using the Macintosh Free Ruler Version 1.6 program. BAEC migration was assessed by comparing distance between wound edges before and after treatment.

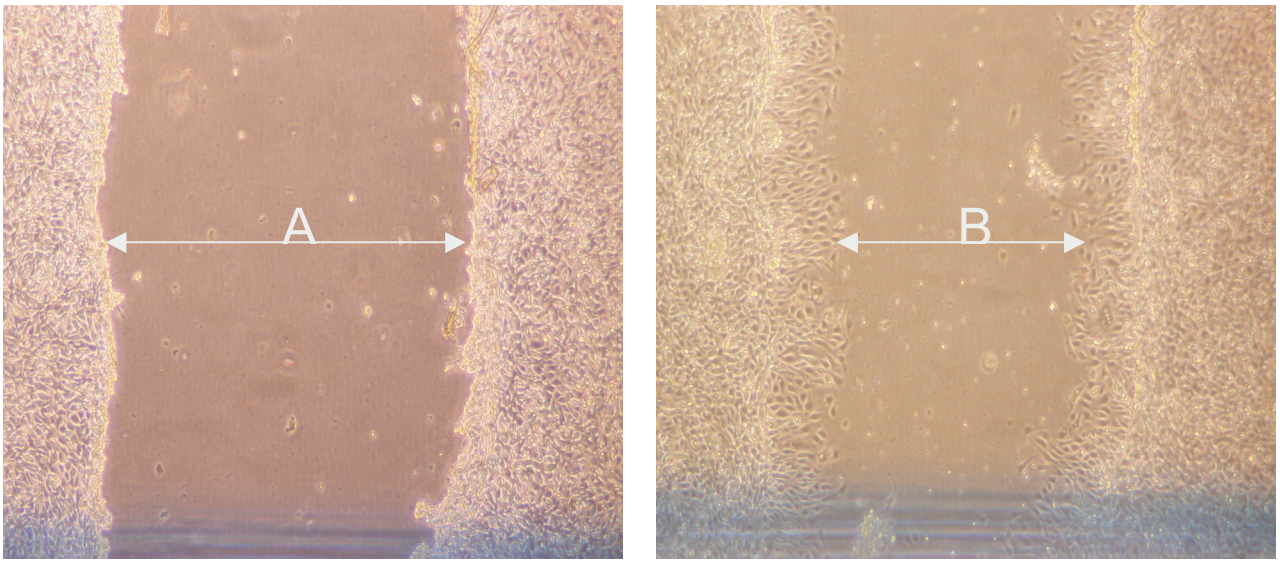


Fig. 2.2.4 A typical tube formation assay. Distance (A) is measured at t=0 h and distance (B) at t=6 h, thus A-B= distance migrated.

2.2.14 2,3-Diaminonaphthalene (DAN) Assay:

This assay measures the concentration of nitrite (downstream product of NO pathway), which accumulates in conditioned media *in vitro* (Misko *et al.*, 1993). *In vivo*, NO is scavenged rapidly and undergoes a series of reactions leaving final

products nitrite (NO_2^-) and nitrate (NO_3^-). Nitrite can subsequently be reacted with 2,3-diaminonaphthalene under acidic conditions to form a fluorescent product naphthalenetriazole. 100 µL of media sample or Nitrite Standards (0-100 pM) was added to a fluorometric plate with 10 µL DAN (0.05 mg/mL in 0.62 M HCl), and incubated for 20 min at 20ºC. 5 µL of 2.8 M NaOH was subsequently added to provide the alkaline pH necessary for measuring naphthalenetriazole fluorescence. Fluorescence was measured using Excitation wavelength: 365nm, Emission wavelength: 450nm and at Slit widths Ex: 10 nm, Em: 7.5 nm using the Perkin Elmer LS50B Luminescent Spectrophotometer model (with plate reader attachment).

2.2.15 Tube Formation Assay

Collagen gels were prepared as previously described (Zheng *et al*., 2001). Briefly, a neutralized collagen mixture was prepared by mixing stock 1.5 mg/mL type I rat tail collagen (in 10 mM acetic acid), with growth medium and 1N NaOH. Typical mixture: 600 µl of collagen + 60 µl of culture medium + 50 µl of 1N NaOH. The mixture was pipetted into 24-well tissue culture plate (100 µl/well) and allowed to gel in an incubator containing 5% CO_2-95% air at 37°C for 1 h. After polymerization the gels were then incubated with growth medium overnight at 37°C before use.

Following 24 h treatment with *HPCM* (0-25% dilutions) or OMP, BAECs were trypsinized, cell counts were performed and $1.5X10^4$ cells were resuspended in

RPMI complete media and seeded in each well of collagen gel. Cells were incubated overnight for 16-18 hours and tube formation monitored using an Olympus SP-350 digital camera. Four random fields of vision were photographed from each gel and length of tube formation was quantified by measuring the length of the network of connected cells in each well (Von Offenberg Sweeney *et al*., 2005) using Olympus Cell* Imaging software for Life Sciences and Microscopy.

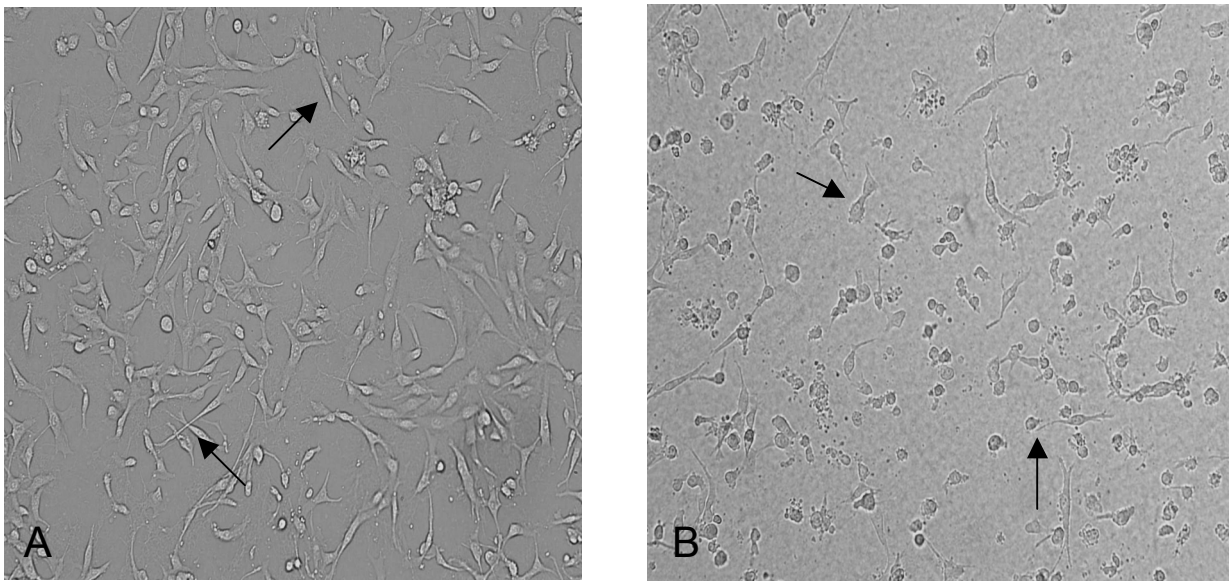


Fig. 2.2.5 A typical tube formation assay. (A) Example of tube length in untreated cells (B) Example of tube length in treated cells. Arrows indicate tubes typically measured.

2.2.16 Statistical Analysis

Results are expressed as mean ±SEM of a minimum of three independent experiments (n=3) unless otherwise stated. Statistical comparisons were performed using unpaired Student's *t*-test or Two-Way ANOVA with replication, where applicable. $^{*}P \leq 0.05$, $^{**}P \leq 0.005$, $^{***}P \leq 0.0005$, $^{\S}P \leq 0.05$, $^{\S\S}P \leq 0.005$, $^{\S\S\S}P \leq 0.0005$ were considered significant.

Chapter 3

Results:

The Effect of Helicobacter pylori Outer Membrane Proteins on Endothelial Cell Properties and Function

3.1 Introduction

H. pylori is a Gram-negative, micro-aerophillic, spiral-shaped bacterium that colonises the human gastric epithelium and is strongly implicated in chronic gastritis, peptic ulcer disease, and gastric carcinoma (Hatakeyama, 2004). It represents one of the most widespread human infectious diseases. Central to the progression of infection and gastric colonization is the bacterium's ability to bind and interact with the epithelial cell surface (Yamaoka *et al.*, 2002). Much research has focused on the how these interactions occur, leading to the identification and characterization of 33 outer-membrane proteins. These proteins are not only responsible for host cell binding, but many have been recognised as virulence factors responsible for the progression of gastric disease.

Recent evidence suggests that infection with *H. pylori* is associated with atherosclerotic plaque formation (Haim Shmuelya *et al.*, 2005, Yamaoka *et al.*, 2002). Injury to the arterial wall resulting in endothelial dysfunction is considered pivotal to the pathogenesis of athersosclerosis, with angiogenesis and apoptosis prominent in plaque formation and rupture. Following the discovery of *H. pylori* DNA in atherosclerotic plaques (Ameriso *et al.*, 2001), it has been postulated that that the OMP's of *H. pylori* may play a role in the progression of atherosclerosis by contributing to endothelial dysfunction.

In this chapter, it was attempted to elucidate the putative contribution of H. pylori to endothelial dysfunction by investigating the angiogenic and apoptotic phenotype of vascular endothelial cells in response to exposure to H. pylori OMP's in vitro.

3.2 Results

3.2.1 Morphology of BAECs, *H. pylori* Outer Membrane Proteins have no effect on the BAEC cytoskeleton

BAEC's were monitored by phase-contrast microscopy (Fig. 3.2.1 a i) and Von Willebrand Factor VIII expression by immunocytochemistry. (Fig. 3.2.1 a ii) Cell's were found to be of typical size and shape with characteristic "cobblestone" morphology, and tested positive for Von Willebrand Factor VIII.

Following BAEC exposure to *H. pylori* OMP (8 μg/mL) for 24 h, no difference was observed in cytoskeletal formation (Fig. 3.2.1 b iii-iv) when compared to untreated control cells (Fig. 3.2.1 b i-ii), as determined immunocytochemically by F-actin staining with rhodamine phalloidin.

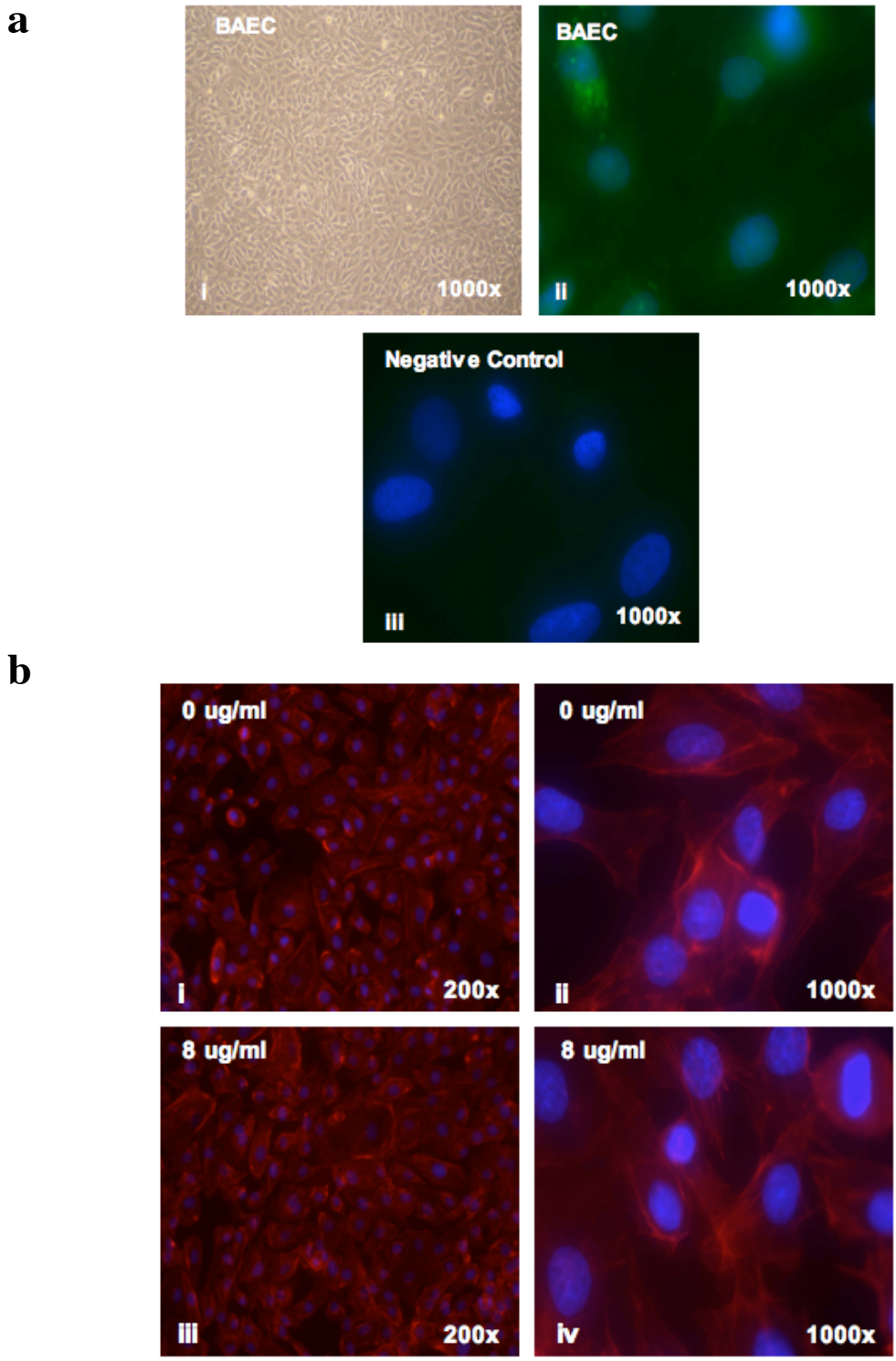


Fig. 3.2.1: Characterization on BAECs and the effect of *H. pylori* OMP on BAEC Cytoskeletal Formation: (a) (i) Endothelial "cobblestone" morphology (ii) Von Willebrand Factor VIII expression (green) (iii) Von Willebrand negative control- no primary antibody (b) Following exposure of BAECs to 8 μg/mL OMP for 24 h, Rhodamine Phallodin expression was examined as above. DAPI stained nuclei are shown in blue. Images are representative.

3.2.2 *H. pylori* Outer Membrane Proteins have no effect on BAEC apoptosis

Following BAEC exposure to increasing concentrations of *H. pylori* OMP (0-8 μg/mL) for 24 h, flow cytometry indicated no increase in apoptosis relative to control (based on positive Annexin V and negative PI binding). Hydrogen peroxide (25 μM) was included as positive control and increased apoptosis by 4.13±0.03 fold relative to control (Fig. 3.2.2a).

Following BAEC exposure to increasing concentrations of *H. pylori* OMP (0-8 μg/mL) and *E. coli* OMP (1 pg) for 24 h no increase in apoptosis relative to control was observed, as determined by Acridine Orange/Ethidium Bromide (AO/EtBr) dual stain. Cells show characteristic green fluorescence (Fig. 3.2.2b i-v), whereas positive apoptotic control cells (Fig. 3.2.2b vi), fluoresce a dark orange colour.

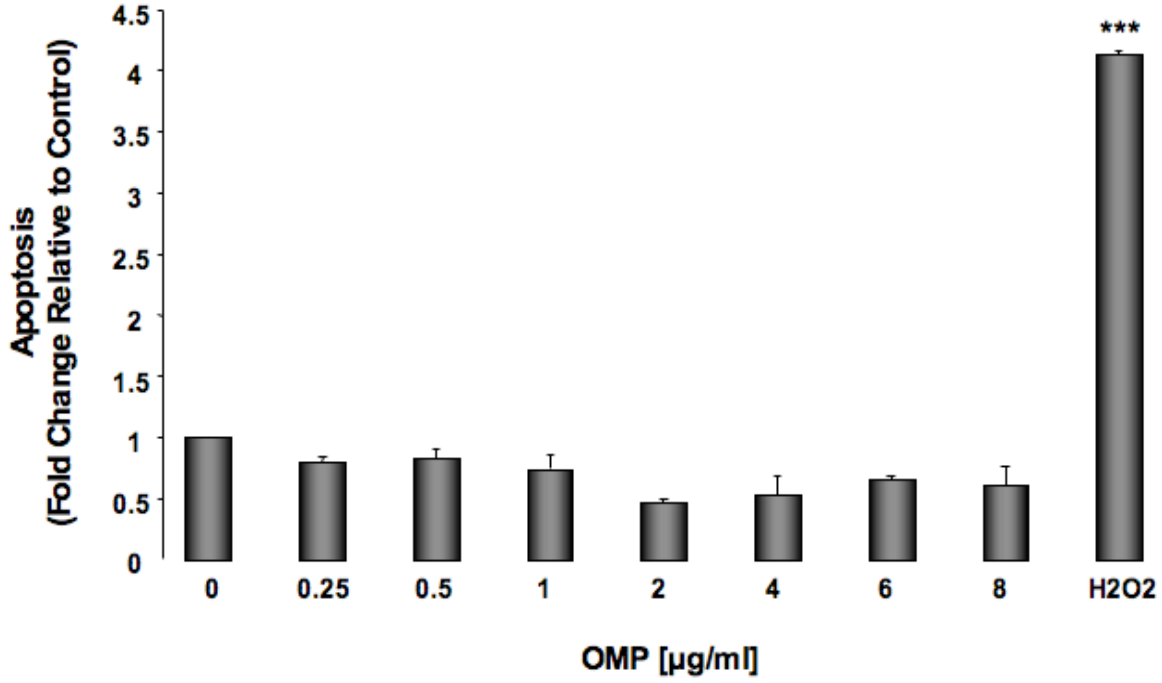


Fig. 3.2.2a: Effect of *H. pylori* OMP on BAEC apoptosis. Following BAEC exposure to OMP (0-8 μg/mL) for 24 h, cells were monitored for apoptosis by (a) FACS analysis. Histograms represent fold change relative to control and are averaged from seven independent experiments ±SEM. Hydrogen peroxide (25 μM H_20_2) included as positive control. ***$P \leq 0.005$ versus control.

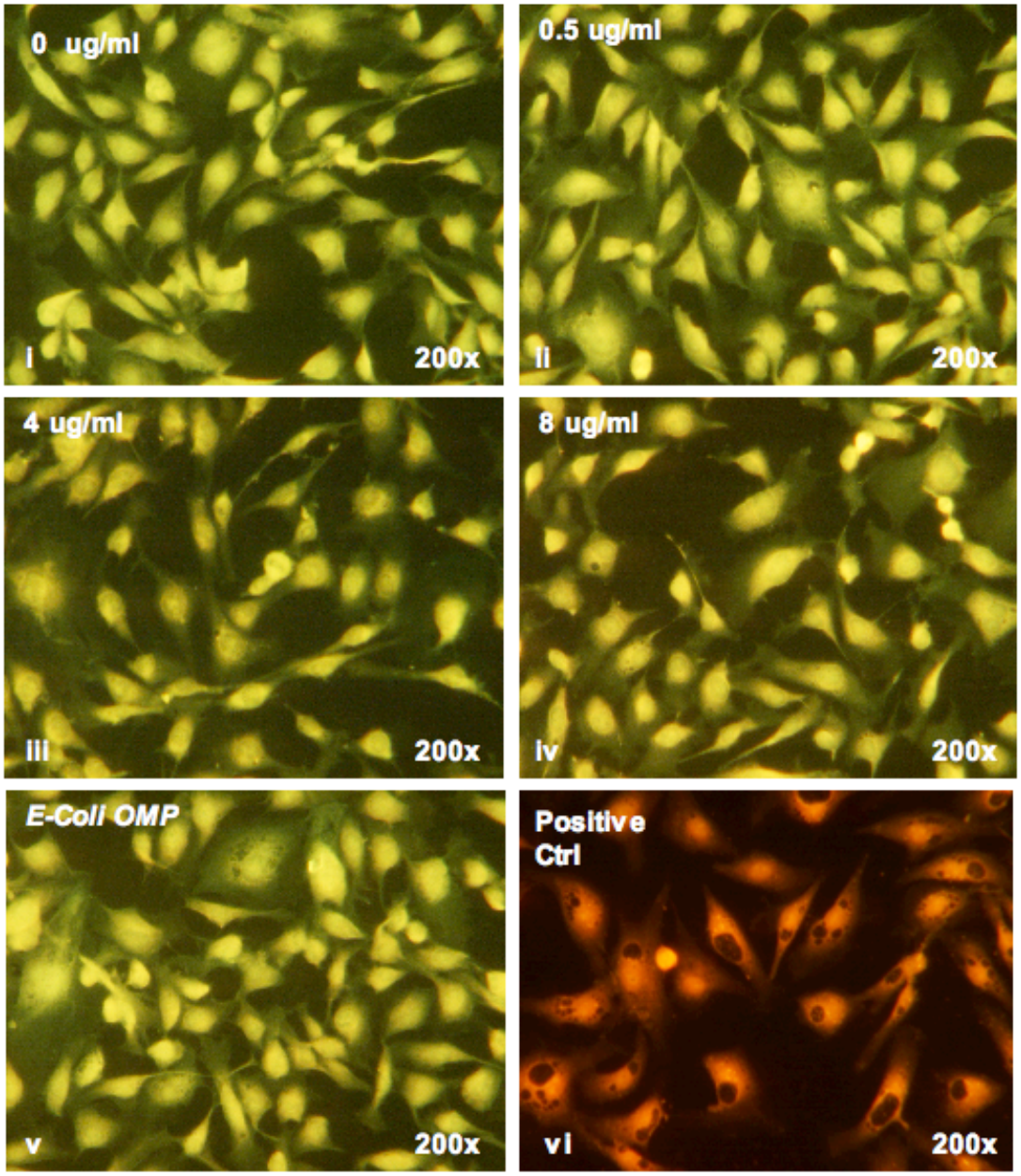


Fig. 3.2.2b: Effect of *H. pylori* OMP on BAEC apoptosis. Following BAEC exposure to *H. pylori* OMP (0-8 μg/mL) for 24 h, cells were monitored for apoptosis by AO/EtBr staining. (i-iv) Increasing OMP concentrations (v) *E. coli* OMP (1 ng) (vi) Positive apoptotic control. Images are representative.

3.2.3 *H. pylori* Outer Membrane Proteins decrease BAEC migration whilst increasing tube formation

Following BAEC exposure to *H. pylori* OMP (0-1 μg/ml) for 24 h, cells were monitored for migration and tube formation. BAEC migration was reduced to 0.71±0.049 and 0.62±0.069 of control at 0.5 and 1 μg/mL *H. pylori* OMP respectively (Fig. 3.2.4 a).

BAEC tube formation was increased to 1.46±0.3 and 1.96±0.58 fold of control at 0.25 and 0.5 μg/mL *H. pylori* OMP respectively (Fig. 3.2.4 b).

3.2.4 *H. pylori* Outer Membrane Proteins have no effect on BAEC proliferation.

Following BAEC exposure to *H. pylori* OMP (8 *μ*g/mL) for 24 h, no change in BAEC proliferation was observed, as monitored by either FACS analysis (Fig. 3.2.4 a ii) or cell counts (Fig. 3.2.4 b).

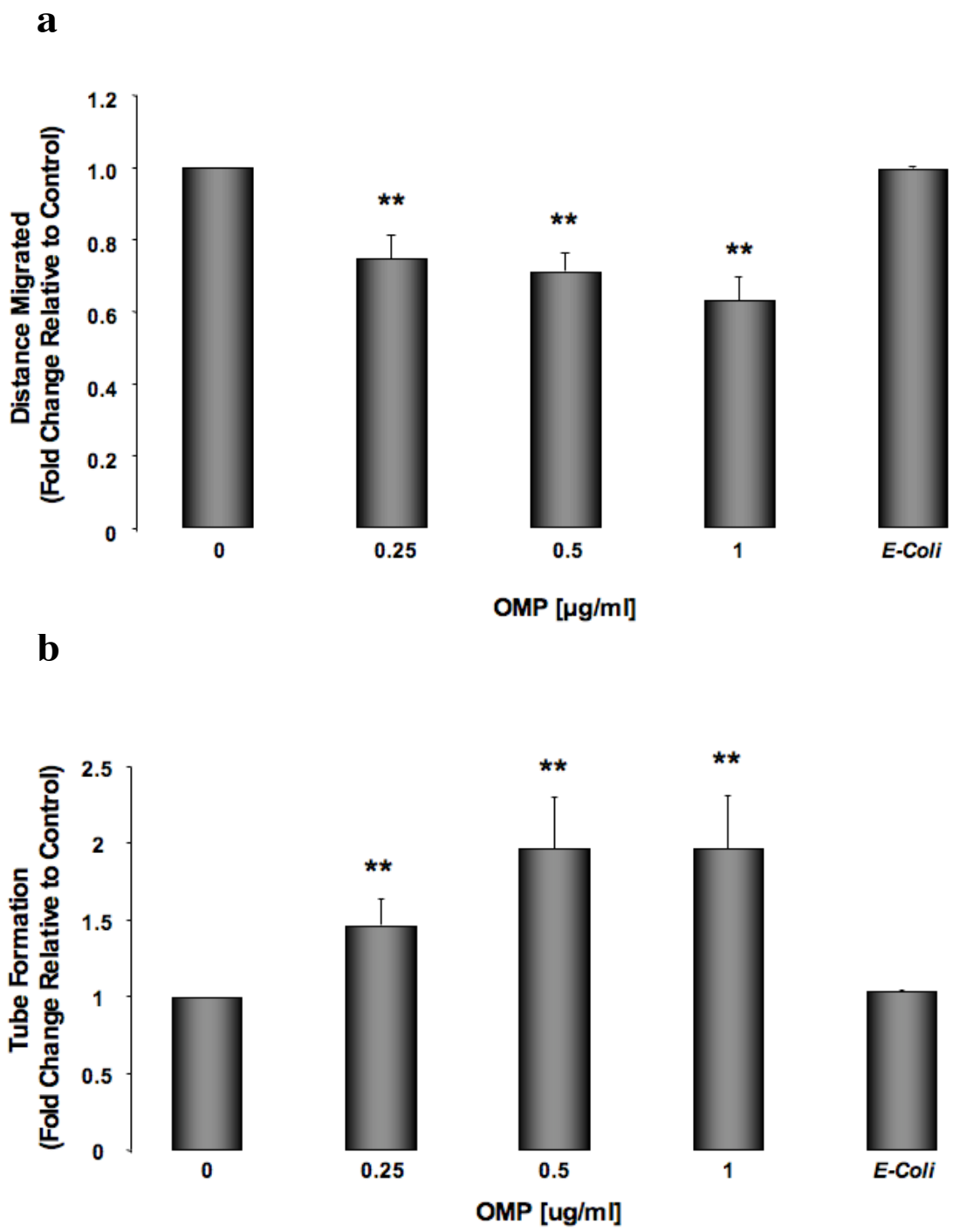


Fig. 3.2.3: Effect of *H. pylori* OMP on BAEC migration and tube formation. Following BAEC exposure to OMP (0-1 μg/mL) for 24 h, cells were monitored for (a) Migration and (b) Tube formation. *E. coli* OMP (1 ng/mL) was included as positive bacterial control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control.

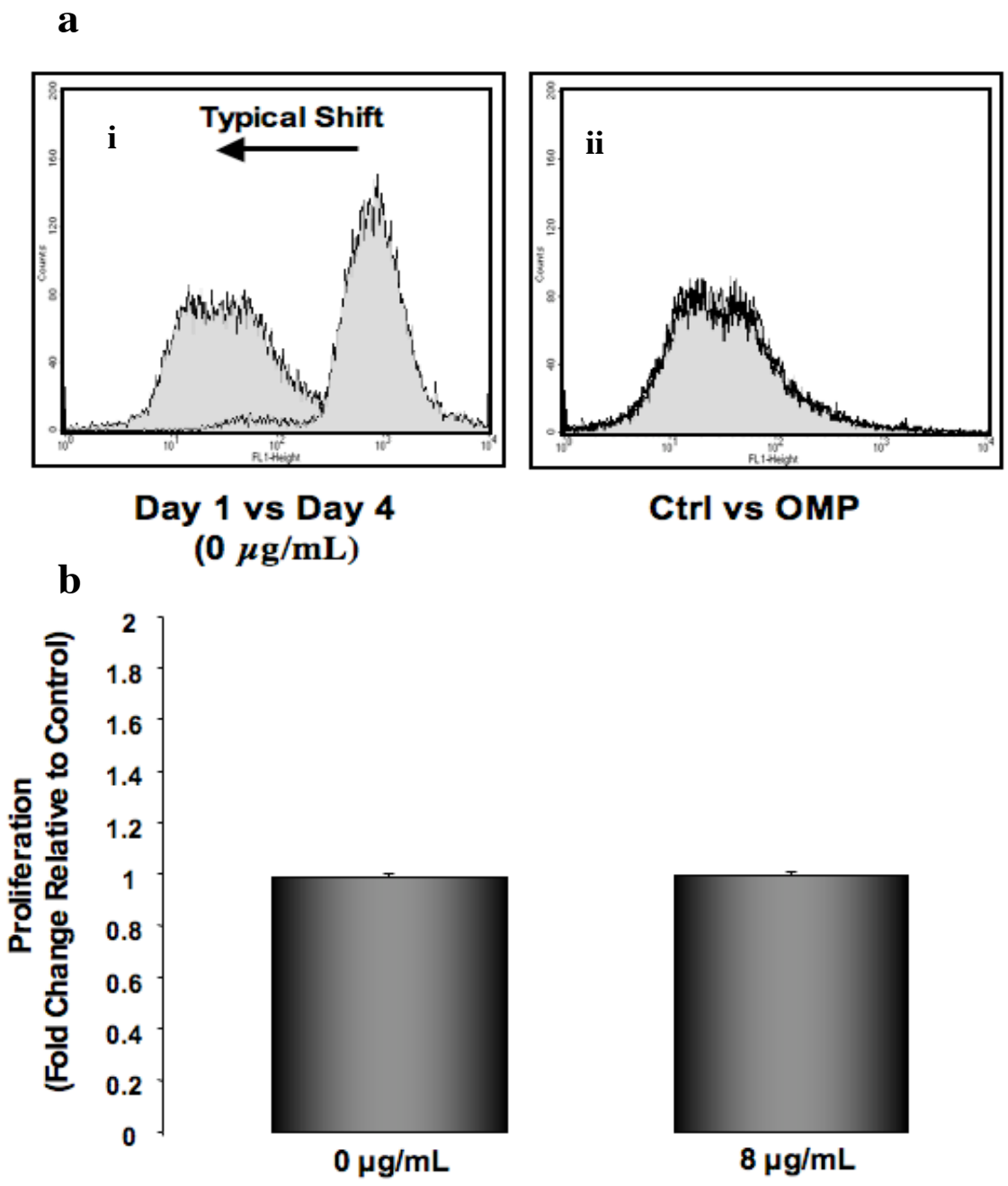


Fig. 3.2.4: Effect of *H. pylori* OMP on BAEC Proliferation. Following BAEC exposure to OMP (8 μg/mL) for 24 h, cells were monitored for proliferation by (a) FACS Analysis- (i) Typical shift in cell body following 4 days proliferation (ii) - grey shaded area represents untreated control cells overlayed with black line representing OMP (b) Cell counts. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM.

3.2.5 *H. pylori* Outer Membrane Proteins have no effect on BAEC ZO-1 localisation.

Following 24 h culture of BAECs in the presence of *H. pylori* OMP (8 μg/mL) and under conditions of 10 dyne/cm^2 laminar shear (upregulates endothelial barrier formation), subcellular localization of ZO-1 within BAEC monolayers was monitored by immunocytochemistry. In sheared cells, OMP treatment saw no change in continuous ZO-1 immunoreactivity along the cell- cell border (3.2.5)

3.2.6 *H. pylori* Outer Membrane Proteins have no effect on BAEC permeability and nitrite production.

Following BAEC exposure to *H. pylori* OMP (8 μg/mL) and 10 dyne/cm^2 laminar shear for 24 h no increase in permeability relative to control (0 μg/mL) was observed, as determined by permeability assay. Unsheared BAECs were included as a positive control and demonstrated slightly increased FD40 Dextran flux of 2.7±0.2 % relative to sheared control cells (Fig. 3.2.6a)

Similarly, no increase in sodium nitrite production (an indicator of cell function) was observed in BAECs incubated with 8 μg/mL OMP for 24 h, as determined by DAN assay. Shearing of BAECs increased sodium nitrite production 2.2±0.07 fold over unsheared control (Fig. 3.2.6b).

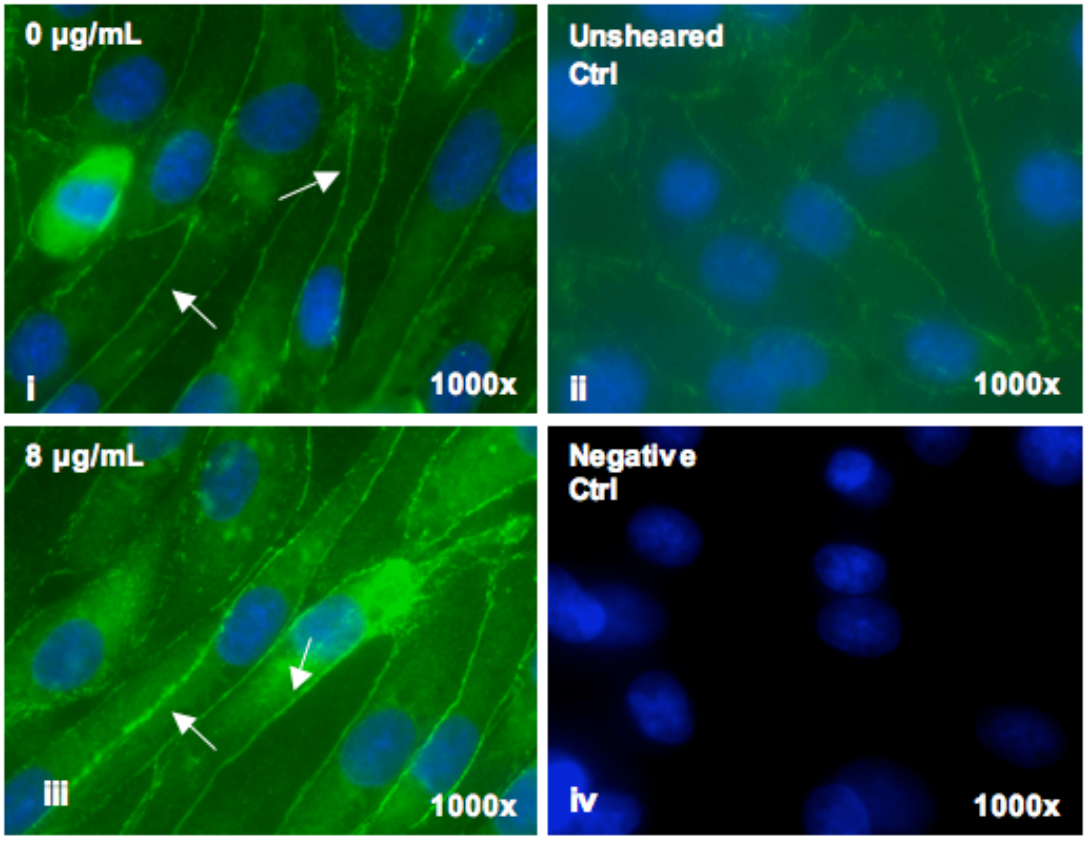


Fig. 3.2.5: **Effect of *H. pylori* OMP on BAEC ZO-1 membrane localization.** Following exposure of cyclically strained BAECs to 8 μg/mL OMP for 24 h, cells were immunocytochemically monitored for ZO-1 localization (i) 0 μg/mL OMP (ii) Unsheared control (iii) 8 μg/mL (iv) Negative control (no primary antibody). White arrows indicate plasma membrane localization of ZO-1. DAPI-stained nuclei (Blue) are clearly visible. Images are representative.

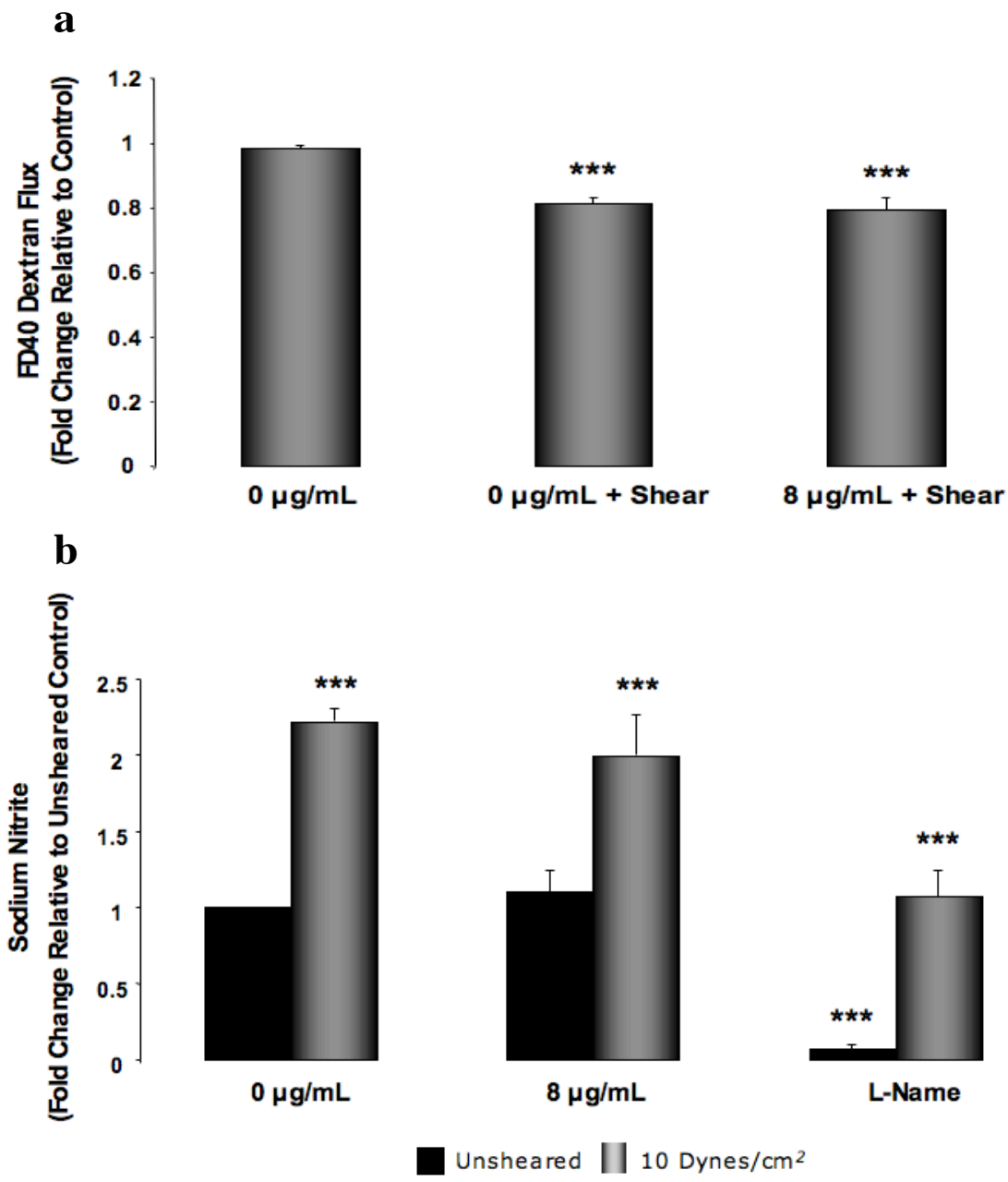


Fig. 3.2.6: Effect of *H. pylori* OMP on BAEC Permeability and Nitrite production. Following BAEC exposure to OMP (8 μg/mL) and shear for 24 h, cells were monitored for (a) Permeability. Histograms represent transendothelial permeability to Dextran after 3 h, and are averaged from three independent experiments ±SEM. (b) Nitrite production. L-Name (1 mM) included as positive control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control.

3.3 Discussion

The outer membrane proteins of *H. pylori* display many properties not commonly found in other bacteria. Its Lipopolysaccharide (Lps) contains fucosylated oligosaccharide antigens that are similar to Lewis antigens, allowing molecular mimicry and eliciting only a weak immune response. In contrast, the Lps of *E. coli* is the governing factor in initiating a strong host immune response (Knapp *et al.*, 2003). Outer inflammatory Protein A (Oip A) acts not only as an adherence protein, but also induces inflammation (Yamaoka et al., 2002). Even the flagella of *H. pylori* differ from many bacteria. Similarly to Lps, they induce only a weak immune response. Importantly, many OMPs are also thought to contribute to gastric injury, and even to the progression of gastric diseases (Cover, 2006). While the function of many of the 33 OMPs are known (see table 3.3.1), much research remains on establishing all effects associated with the proteins. For the above reasons, the OMPs represent an interesting area of study with many possibilities and potential roles in not only altering the cell to which they interact with, but also inducing disease states.

Numerous studies have reported a link between *H. pylori* and atherosclerosis (Grabczewska *et al.*, 2006, Grebowska *et al.*, 2006, Kaplan *et al.*, 2006, Triantafilou *et al.*, 2007). Central to these studies is the possibility that endothelial dysfunction, resulting from an interaction between the bacterium and the endothelium, can initiate and even progress the formation of an atherosclerotic plaque. To the author's knowledge little or no research has been carried out on the effect of *H. pylori* OMP's directly on endothelial cells. To this end, endothelial properties and function were investigated following exposure to a *H. pylori* OMP fraction.

Protein/gene cluster	Predicted role	Association with *H. pylori*-related disease	Reference(s)
BabA	Binds to fucosylated Le^b blood group antigen on cells	*babA2* allele has been implicated in peptic ulcer disease and gastric cancer	63, 221, 246, 285, 511, 516, 702, 703
SabA	Binds to sialyl-Le^x and sialyl-Le^a antigens and is involved in activation of neutrophils	None	117, 387, 639
SabB	Binding specificity is unknown	Absence of SabB expression via phase variation is associated with duodenal ulcers	117
OipA	OipA has been reported to assist in IL-8 induction, but this association is not universal	Expression of OipA is linked to *cag* status and development of duodenal ulcers and gastric cancer	16, 17, 246, 330, 705, 706
AlpA and AlpB	Inactivation of the *alpA* and *alpB* genes results in decreased adherence to gastric epithelial cells and absence of colonization in a guinea pig model	Unknown	114, 467, 470
HP-NAP	HP-NAP is reported to activate neutrophils and is a possible adhesin to mucin; possible function in protection of *H. pylori* DNA or iron storage	Unknown	172, 438, 439, 452, 631
Plasticity region (*jhp0947–jhp0950*)	Unknown	Presence of the plasticity region is associated with development of gastric cancer, MALT lymphoma, and duodenal ulcers	84, 115, 462, 463, 545
IceA	The *iceA1* allele encodes a CATG-recognizing restriction endonuclease	IceA1 has been associated with peptic ulcer disease, but this association is not universal	129, 185, 313, 495, 648, 700, 704
DupA	The *dupA* gene encodes a VirB4 ATPase homolog	Associated with duodenal ulcers but also with reduced risk for gastric atrophy and cancer	376

Table 3.3.1. Adhesions and virulence-associated proteins of *H. pylori*. (Kusters et al., 2006)

Initial investigations focused on whether OMP has any effect on the BAEC cytoskeleton by examining actin expression. Since actin participates in a host of cellular functions such as muscle contraction, cell division, cell signalling, cell junction maintenance and cell shape, any alteration in actin dynamics may indicate a change in cell function. However, no such alteration was observed in response to OMP treatment. Previous studies have indicated that both the CagA and VacA proteins of the bacterium are responsible for actin rearrangements in gastric cells (Hennig *et al*., 2005, Selbach *et al*., 2003). Hence, this result potentially points to the purity of our OMP preparation, with no VacA or CagA present. We next determined whether OMP had any effect on BAEC apoptosis, but similar to results found in

epithelial cells (Toyoda *et al.*, 2005), no effect was shown with concentrations of up to 8 μg/mL OMP.

In order to examine the angiogenic phenotype of our cells in response to *H. pylori* we looked at BAEC migration and tube formation, processes that are central not only in angiogenesis, but to maintenance of a healthy endothelium. Interestingly, incubation with OMP decreased BAEC migration, whilst increasing tube formation, In an atherosclerotic phenotype for example, where endothelial dysfunction predominantes and the endothelium is unable to sufficiently repair a vascular injury, it would be expected that both tube formation and migration are decreased. Due to lack of comparable data, it can only be speculated as to why we observed opposing effects. One possibility may be that the integrins involved in migration (as discussed in Section 1.2.2.2) are not sufficiently stimulated after 24 h to initiate migration. Cell migration speed is limited by the rate of integrin attachment at the front of the cell and detachment at the cell rear. Since integrin activation, binding and disassociation, follow a bell-shaped curve (Palecek *et al.*, 1999), it is conceivable that after 6-8 h, the integrins, following an "all or nothing" migratory response may not yet have reached their peak rate of disassociation and thus no positive migration relative to control is occurring, however this does not explain the decrease we have observed. A second theory is the possibility that, as many functions of the outer membrane proteins remain unclear there may simply be anti-migratory or pro-angiogenic stimuli in the fraction preparation. Finally, as this model was conducted in an *in vitro* system which would differ to the exact manner in which OMP's might come into contact with the endothelium *in vivo* the result may be artifactual. Further

studies may clarify whether any of these theories are correct. An experiment examining the activation profile of the integrins involved in migration over time should yield a clearer answer on their involvement. Moreover, isolation and testing the functions of individual OMP's in an *in vivo* model, would greatly enhance our understanding of their actions and may allow determination of which protein(s) in particular are causing these opposing effects.

Further investigation also revealed that OMP has no effect on BAEC proliferation. This is similar to the findings of Toyoda *et al.,* where OMP was shown to have no effect on cell cycle of gastric cancer cell lines (MKN45 cells) (Toyoda *et al*., 2005). In addition to the examination of cell properties, the effect of OMP on vascular endothelial barrier integrity was also monitored. Previous work by our group has demonstrated endothelial barrier up-regulation in response to cyclic circumferential strain and shear stress, in BAECs (Collins *et al*., 2006) and bovine brain microvascular endothelial cells (BBMvECs) (Colgan et al., 2007b), respectively. For these studies, immunoreactivity of tight junction zonnula occludens-1 (ZO-1) along the cell-cell border was successfully employed as an index of barrier integrity. Following incubation of BAECs with OMP, ZO-1 localization was well defined and continuous along the cell-cell border in both untreated and treated cells. These results indicate an intact and fully functional barrier. Consistent with these ZO-1 stain findings, an FD-40 Dextran permeability assay was also employed and flux of FD40 across a cell monolayer did not change in response to OMP treatment.

The role of both proliferation and barrier function in atherosclerosis has been well documented. In particular, a weakened barrier resulting in an influx of lipoproteins, is a hallmark of atherosclerotic plaque initiation (Peters *et al*., 2007). Since no change in either proliferation or permeability is occurring following incubation with OMP, it would suggest that these proteins may not contribute to the endothelial dysfunction associated with vascular disease. To add further weight to this, nitrite production by BAECs was examined following incubation with OMP. As previously discussed (section 1.2), attenuation of NO is one of the earliest biochemical changes preceding endothelial dysfunction. No change in BAEC nitrite levels was found after incubation with OMP.

These results, when viewed collectively suggest that *H. pylori* OMP has relatively minor effects on BAEC properties and function *in vitro*. This further suggests a limited role for these proteins with respect to endothelial dysfunction *in vivo*. While it is possible that OMP's directly interact with the endothelium, there was no evidence they interfere with a cell's functional properties in a pro-atherogenic manner *in vitro*. Moreover, it may be necessary for the intact bacterium to be present forming an integrated response for OMP virulence to be fully realised. However, it is important to note that studies have identified other mechanisms by which OMPs could in fact contribute to the progression of an atherosclerotic phenotype. OipA is one such mechanism, as it has been shown to promote inflammation (Yamaoka *et al*., 2000)- a key event in atherosclerosis. In addition, Triantafilou *et al.* have indicated that the Lps from *H. pylori* can induce inflammatory responses through toll-like receptor 2 in human endothelial cells

(Triantafilou *et al.*, 2007). Alp A and B have been identified as important in adherence to epithelial cells resulting in gastric colonization and have even been identified for use in vaccines against *H. pylori* (de Jonge *et al.*, 2004).

In conclusion, *H. pylori* OMPs have the ability to influence BAEC migration and tube formation, without affecting proliferation, apoptosis or barrier function. From published data, it is possible that if OMPs are involved in atherosclerosis they may play two key roles. Firstly, they facilitate adherence of the bacterium to endothelial cells (as they do in epithelial cells). Following adhesion, the bacteria can inject highly virulent molecules such as CagA, or secrete VacA, both of which have profound effect on the endothelium. Secondly, OMP's have the ability to stimulate production of inflammatory cytokines such as Il-8 (Yamaoka et al., 2002), hence exacerbating or even initiating a vascular lesion. It is also worth noting that *H. pylori* has the ability to "shed" its OMPs. This means that the bacteria itself would not have to be in contact with the endothelium, but its released components could travel in the bloodstream and interact with the vessel wall. This, coupled with the ability of OMPs to induce inflammation and govern adherence to host cells, means a role for *H. pylori* OMP's in atherosclerotic plaque initiation or development cannot be discounted. However, from these studies the link appears unlikely.

Chapter 4

Results:

The Effect of Helicobacter pylori Conditioned Media on Endothelial Cell Properties and Function

4.1 Introduction

Seroepidemiological and eradication data suggest a casual relationship between *H. pylori* infection and atherosclerosis/coronary heart disease (Ando *et al*., 2006, Grabczewska *et al*., 2006). Elevated levels of homocysteine (Corrado and Novo, 2005), asymmetric dimethylarginine (Marra *et al*., 2005) and serum lipids (Laurila *et al*., 1999) associated with *H. pylori* infection have all been presented as possible contributors to atherogenesis. Importantly, *H. pylori*-specific DNA and VacA cross-reactive antigens have been detected in atherosclerotic plaques (Kowalski *et al*., 2002), suggesting a direct interaction of the bacterium with vessel wall, and potentially the initiation of an atherosclerotic phenotype.

Studies indicate that the role of *H.pylori* in atherosclerosis may derive from its effects on endothelial cell fate and barrier function. Incubation of microvascular endothelial cells with *H.pylori* extracts, or direct co-culture with the bacterium, has been shown to decrease cell viability (Kalia *et al*., 2001) and inhibit angiogenesis (Jenkinson *et al*.). A recent study by Pearce *et al.* has shown that *H.pylori* extracts can inhibit proliferation in both human dermal microvascular endothelial cells (HDMvECs) and human umbilical vein endothelial cells (HUVECs) (Pearce *et al*., 2004), whilst studies by Kurosawa *et al.* demonstrate that *H.pylori* extracts inhibit HUVEC proliferation and induce apoptosis in a CagA/VacA-independent manner (Kurosawa et al., 2002). Other recent studies have also shown that *H.pylori*-conditioned medium and co-culture significantly increase neutrophil recruitment and transendothelial migration (Enarsson et al., 2005).

Despite these observations, the issue of *H.pylori* infection and atherosclerosis is still controversial, the precise mechanism(s) of microbial translocation from gastric epithelium to arterial lumen as yet to be determined. Vascular endothelial cell models employed are quite diverse, yielding data of limited mechanistic value. Moreover, to our knowledge, no studies of this type have been performed in endothelial cells of aortic or coronary artery origin, where atherosclerosis typically predominates.

In this chapter, to help address this knowledge deficit, the effects of H.pylori conditioned medium on bovine aortic endothelial cell (BAEC) properties and function was investigated.

4.2 Results

4.2.1 *HPCM* alters BAEC morphology and causes cell vacuolation.

Following BAEC exposure to *HPCM* (0-100%) for 24 h, cell morphology and vacuolation were examined by phase-contrast microscopy. Incubation of BAECs with *HPCM* concentrations of 50% and greater induced extensive vacuole formation (Fig. 4.2.1 iii-viii), and a change in cell shape to an elongated phenotype- commonly referred to as a "hummingbird" phenotype. These effects were not present at or below *HPCM* concentrations of 25% (Fig. 4.2.1 ix-x).

4.2.2 *H. pylori* Conditioned Media alters BAEC cytoskeletal formation.

Following BAEC exposure to *HPCM* (0-100%) for 24 h, actin cytoskeletal formation was examined immunocytochemically by staining with rhodamine phalloidin. Incubation of BAECs with *HPCM* concentrations of 25% and greater (Fig. 4.2.2 iii-x) revealed significant increase in actin expression and stress fibre distribution.

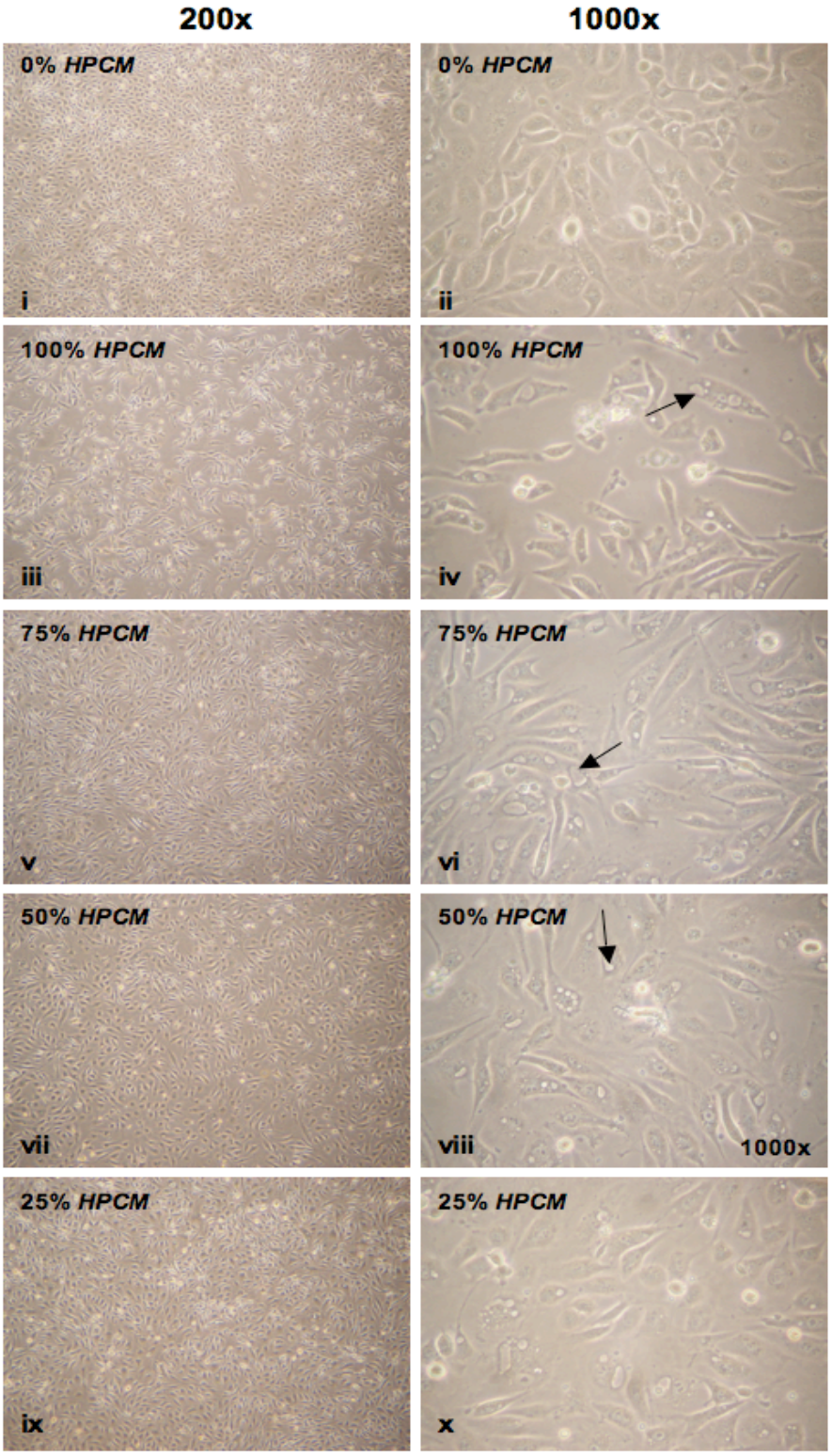


Fig. 4.2.1: Effect of *HPCM* on BAEC morphology. Following BAEC exposure to HPCM (0-100%) for 24 h, cell morphology was examined by phase contrast. (i-ii) 0% *HPCM* (iii-x) Decreasing *HPCM* dilutions. Black arrows indicate vacuolation. Images are representative.

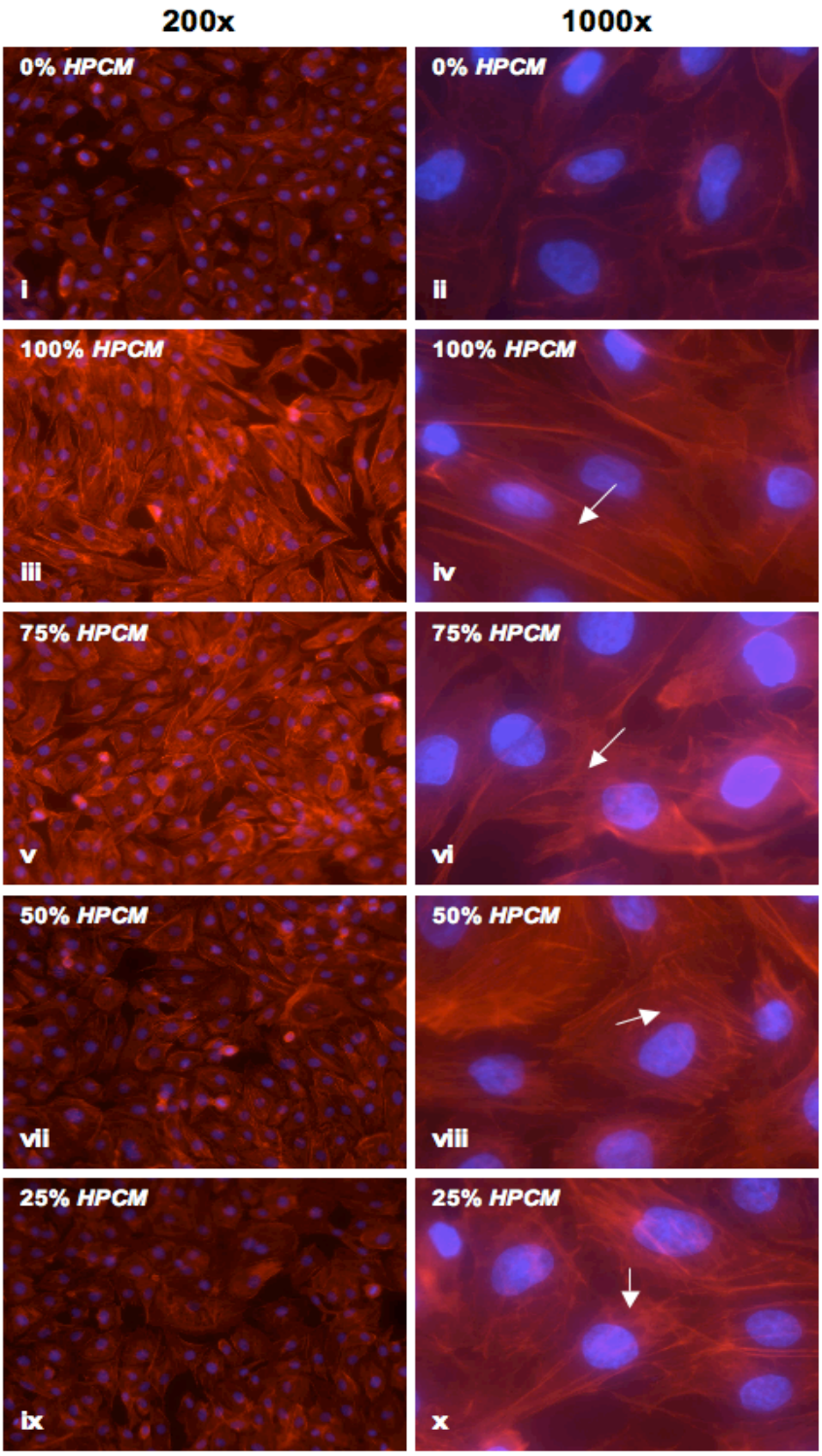


Fig. 4.2.2: Effect of *HPCM* on BAEC cytoskeletal formation. Following BAEC exposure to HPCM (0-100%) for 24 h, actin cytoskeletal formation was examined by Rhodamine-Phallodin expression using standard fluorescent microscopy. (i-ii) 0% *HPCM* (iii-x) Decreasing *HPCM* dilutions. White arrows indicate actin-filaments. Images are representative.

4.2.3 *HPCM* increases BAEC apoptosis in a dose-dependent manner.

Following BAEC exposure to *HPCM* (100%) for 24 h, apoptosis increased by 8.05±0.65 fold over control, as determined by flow cytometry. This figure decreased dose-dependently to 2.75±0.1 fold at 50% *HPCM*, whilst lower concentrations showed no significant increase (Fig. 4.2.3 a).

Similar dose-dependent trends were observed by Caspase-3 assay, with 2.37±0.38 and 1.14±0.06 fold increases in apoptosis over control at 100% and 50% *HPCM* respectively (Fig. 4.2.3 b).

Following BAEC exposure to *HPCM* (0-100%) for 24 h, BAEC apoptosis was examined by AO/EtBr staining. *HPCM* concentrations of 25% and above displayed a dose-dependent increase in orange fluorescence- an apoptotic indicator (Fig. 4.2.4 iii-iv). For subsequent experiments *HPCM* dilutions at or below 25% were employed.

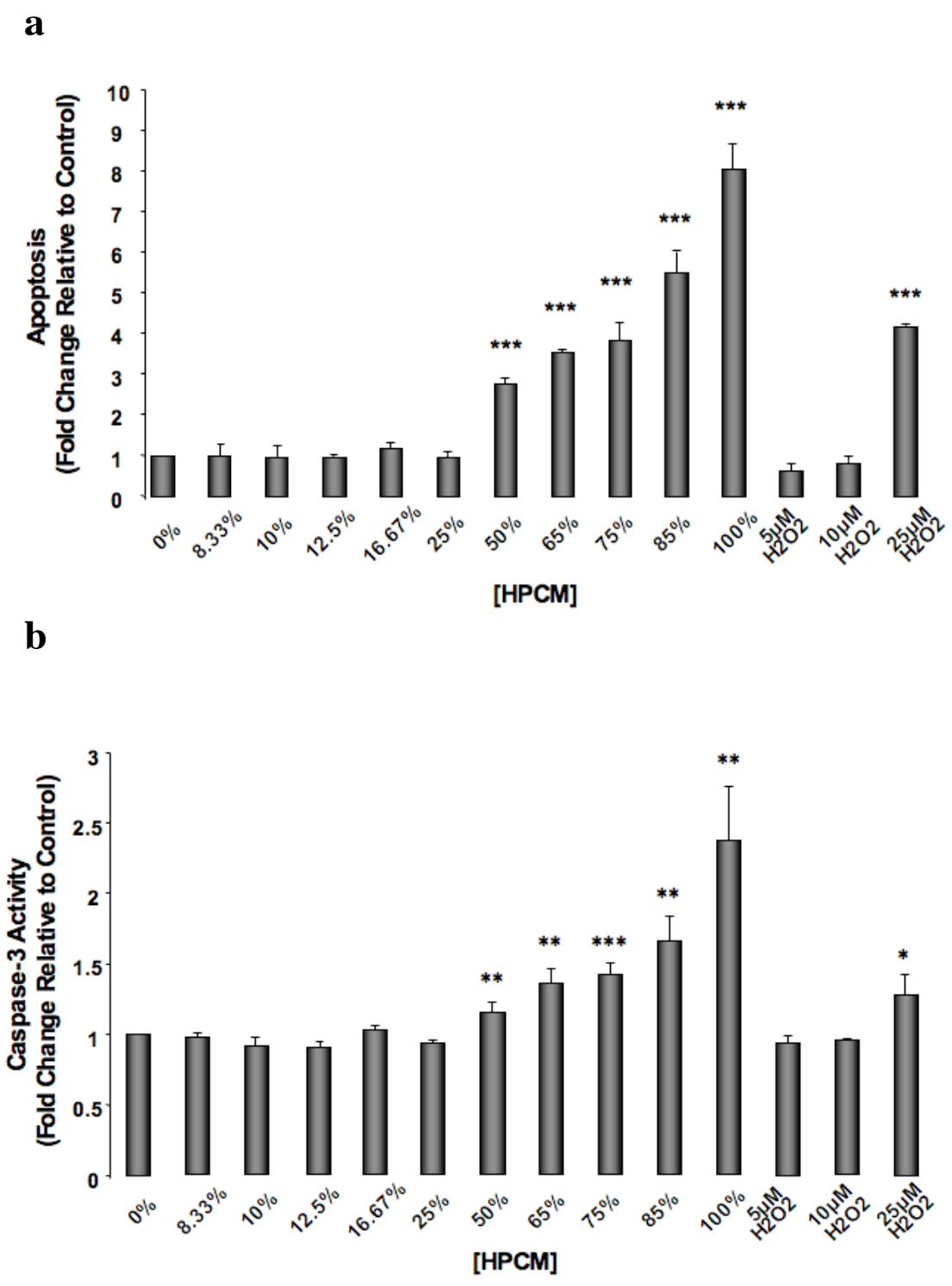


Fig. 4.2.3: Effect of *HPCM* on BAEC apoptosis. Following BAEC exposure to HPCM (0-100%) for 24 h, cells were monitored for apoptosis by (a) FACS analysis and (b) Caspase-3 assay. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. Hydrogen peroxide (5, 10 and 25 μM H_20_2) included as positive control. *P≤0.05 versus control. **P≤0.005 versus control. ***P≤0.0005 versus control.

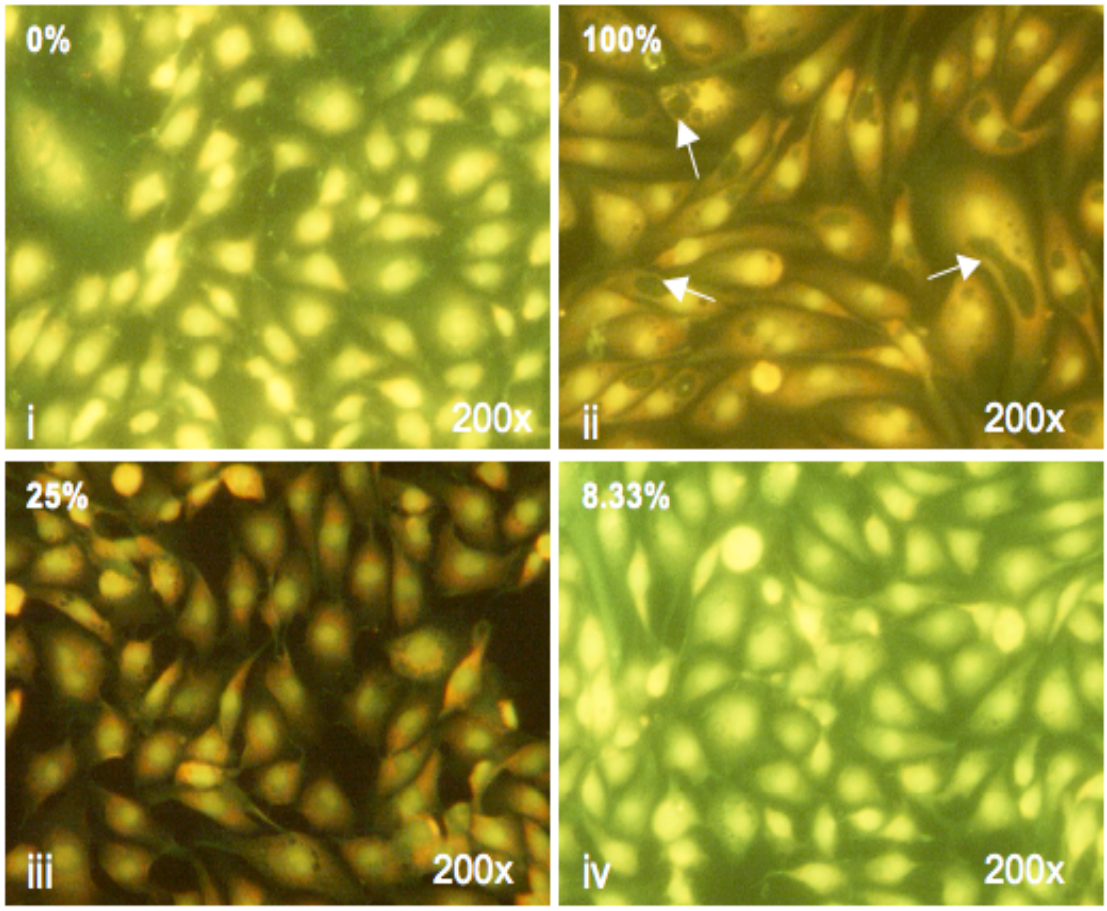


Fig. 4.2.4: Effect of *HPCM* on BAEC apoptosis. Following BAEC exposure to HPCM (0-100%) for 24 h, cells were monitored for apoptosis by AO/EtBr staining. (200x). (i) 0% *HPCM*, (ii) 100% *HPCM*, (iii) 25% *HPCM*, (iv) 8.33% *HPCM*. White arrows indicate vacuoles. Images are representative.

4.2.5 *HPCM* decreases BAEC migration and tube formation in a dose-dependent manner.

Following BAEC exposure to *HPCM* (0-25%) for 24 h, cells were monitored for migration and tube formation. BAEC migration was dose-dependently reduced to 0.69±0.02 fold of control at 25% *HPCM* (Fig. 4.2.5 a), whilst BAEC tube formation was dose-dependently reduced to 0.67±0.04 fold and 0.37±0.02 fold of control at 12.5 and 25% *HPCM*, respectively (Fig. 4.2.5 b). *E. coli*- conditioned media was included in both experiments as a Gram-negative bacterial control and had no effect on migration or tube formation.

4.2.6 *HPCM* decreases BAEC proliferation in a dose-dependent manner.

Following BAEC exposure to *HPCM* (0-25%), cells were monitored for proliferation over 4 days. A dose-dependent decrease in BAEC proliferation with increasing *HPCM* concentration was observed by FACS analysis (Fig. 4.2.6a).

Similarly, at 25% *HPCM*, BAEC proliferation decreased to 0.57±0.01 fold of control, as determined by cell counts (Fig. 4.2.6b). *E. coli*- conditioned media was included in both experiments as a gram-negative bacterial control and had no effect on proliferation.

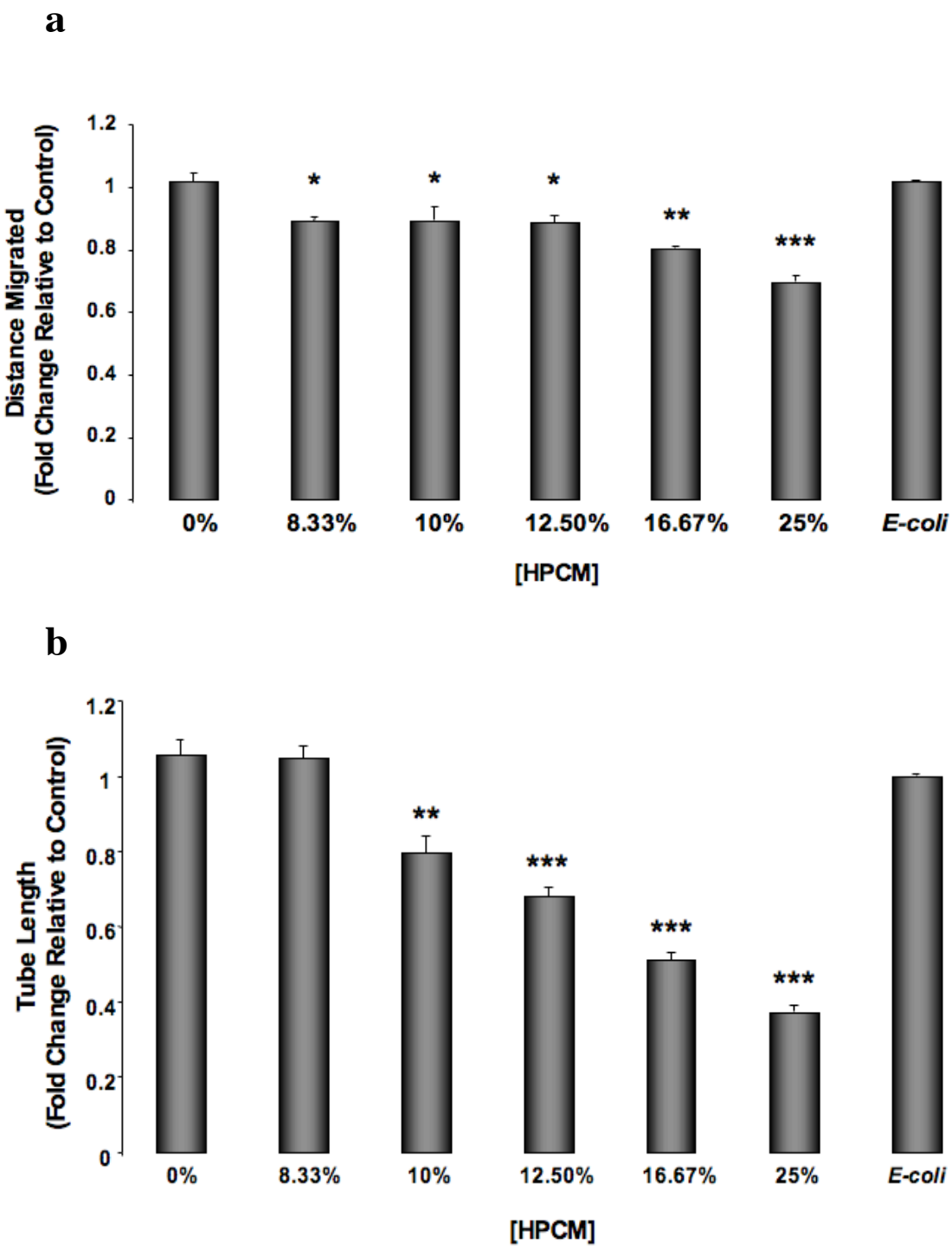


Fig. 4.2.5: Effect of *HPCM* on BAEC Migration and Tube Formation. Following BAEC exposure to HPCM (0-25%) for 24 h, cells were monitored for (a) Migration and (b) Tube formation. *E. coli* conditioned media was included as gram-negative bacterial control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. *$P \leq 0.05$ versus control. **$P \leq 0.005$ versus control. ***$P \leq 0.0005$ versus control.
control.

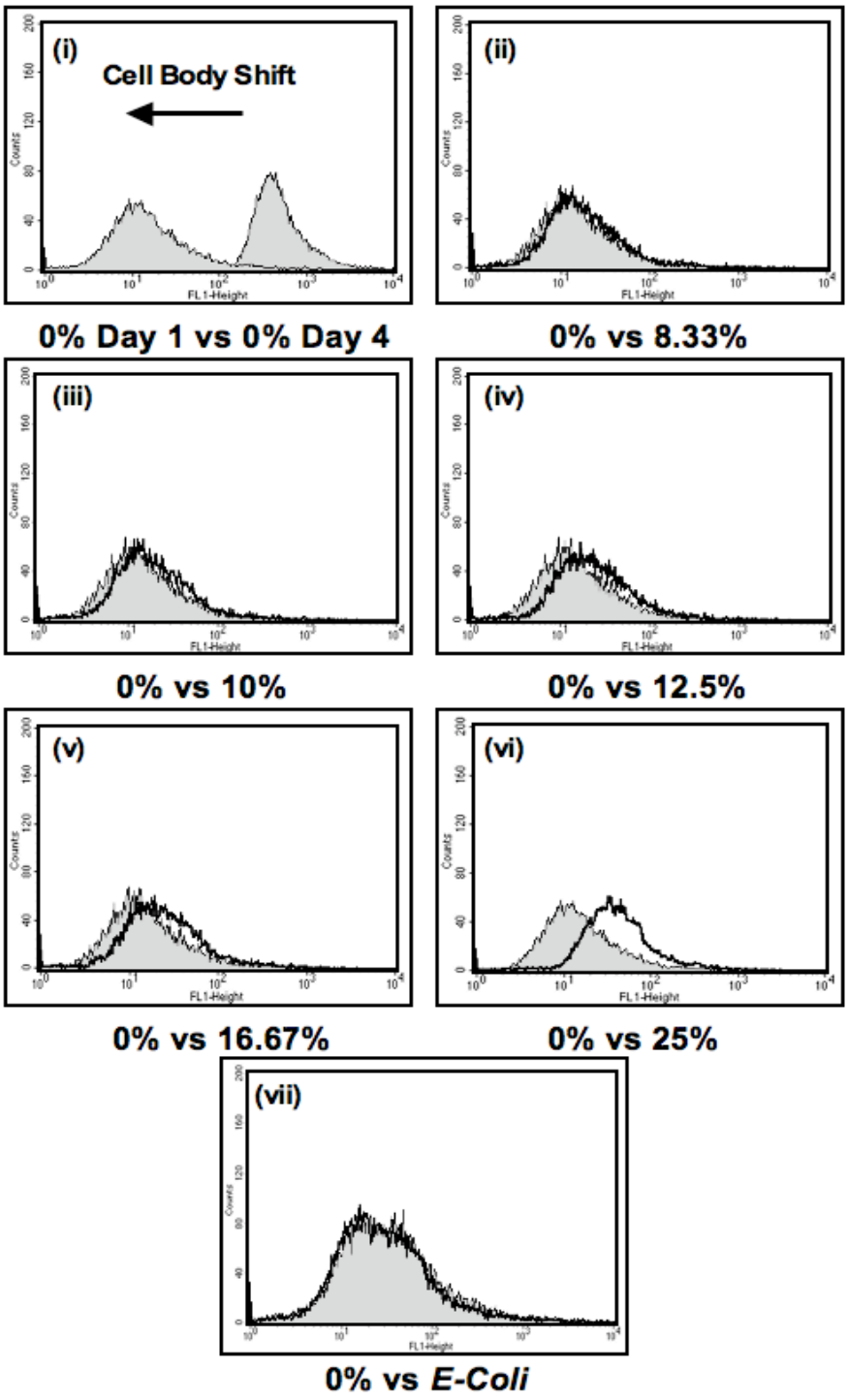


Fig. 4.2.6a: Effect of *HPCM* on BAEC Proliferation. Following BAEC exposure to HPCM (0-25%) for 4 days, proliferation was monitored by FACS Analysis. (i) Arrow shows shift in cell population after 4 days (ii) 0 vs. 8.33% (iii) 0 vs. 10% (iv) 0 vs. 12.5% (v) 0 vs. 16.67% (vi) 0 vs. 25% (vii) 0 vs. *E. coli*. Grey shaded area represents untreated control cells whilst black line represents *HPCM* treated cells. *E. coli* conditioned media was included as gram-negative bacterial control. Curves are representative.

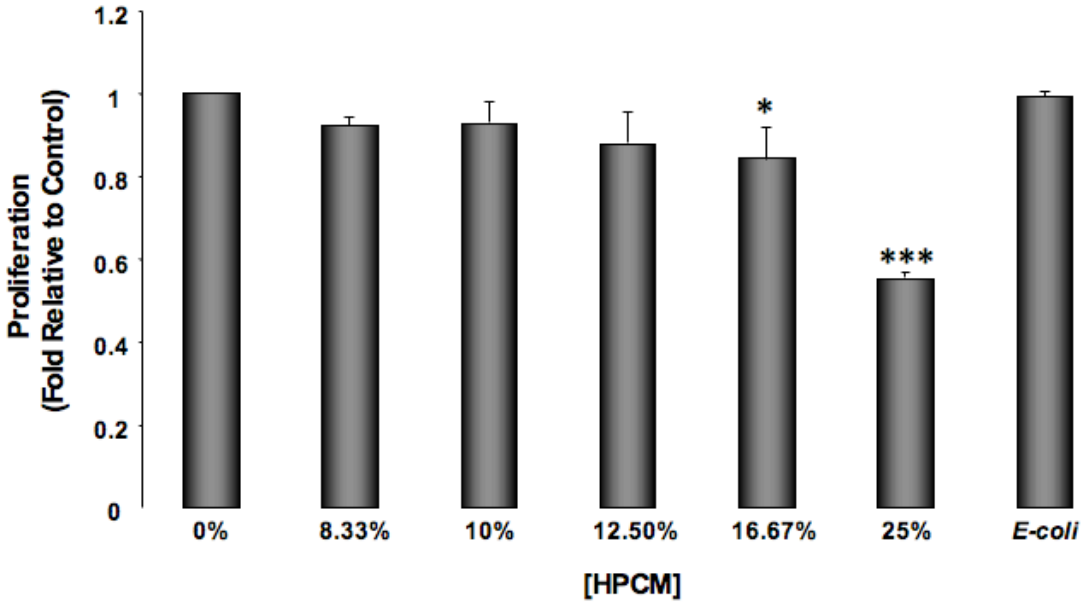


Fig. 4.2.6b: Effect of *HPCM* on BAEC Proliferation. Following BAEC exposure to HPCM (0-25%) for 4 days, proliferation was monitored by cell counts. *E. coli* conditioned media was included as a gram-negative bacterial control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control.

4.2.7 NPPB blocks the *HPCM*-mediated decrease in BAEC tube formation and migration.

Following BAEC exposure to 25% *HPCM* for 24 h in the absence and presence of NPPB, cells were monitored for migration and tube formation. NPPB is an inhibitor known to selectively block the chloride channels through which VacA elicits its effects. Exposure of cells to NPPB reduced baseline migration to 0.95±0.08 fold of control, whilst 25% *HPCM* reduced tube formation to 0.75±0.011 fold of control. Exposure of BAECs to 25% *HPCM* in the presence of NPPB completely reversed the effect of *HPCM* on BAEC migration (Fig. 4.2.7 a). Furthermore, exposure of cells to 25% *HPCM* also reduced tube formation to 0.39±0.008 fold of control, an effect that was completely reversed by treatment with NPPB (Fig. 4.2.7 b). Urease (which is also found in *HPCM*) was included in both experiments to elucidate its role on BAEC function. No effect was observed following incubation with the enzyme on BAEC migration or tube formation.

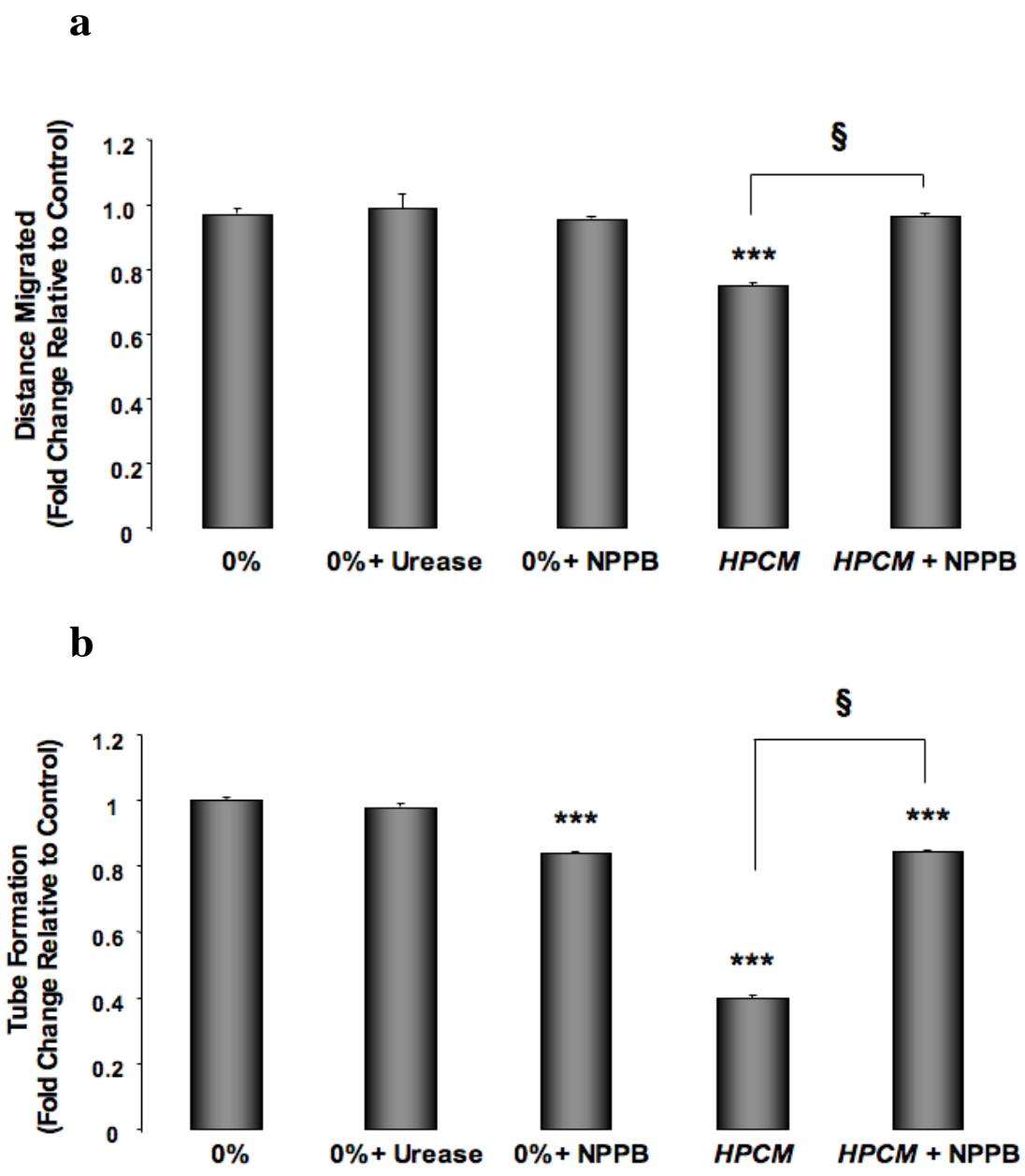


Fig. 4.2.7: Effect of NPPB on HPCM-induced changes in BAEC tube formation and migration. Following BAEC exposure to 25% HPCM for 24 h in the absence and presence of 100 mM NPPB, cells were monitored for (a) Tube formation and (b) Migration. Histograms are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus 25% *HPCM*.

4.2.8 NPPB blocks the *HPCM*-mediated decrease in BAEC proliferation.

Following BAEC exposure to 25% *HPCM* in the absence and presence of NPPB, cells were monitored for proliferation over 2 days by FACS Analysis. As previously shown, 25% *HPCM* reduced proliferation relative to control, however inclusion of NPPB in 25% *HPCM* completely reversed the effect of *HPCM* on BAEC proliferation. Urease had no effect on proliferation (Fig 4.2.8a).

Similarly, cell counts revealed that exposure of cells to NPPB reduced baseline proliferation to 0.82±0.01 fold of control, whilst 25% *HPCM* reduced proliferation to 0.64±0.01 fold of control. Exposure of BAECs to 25% *HPCM* in the presence of NPPB completely reversed the effect of *HPCM* on BAEC proliferation. Urease had no effect on proliferation relative to control (Fig 4.2.8b).

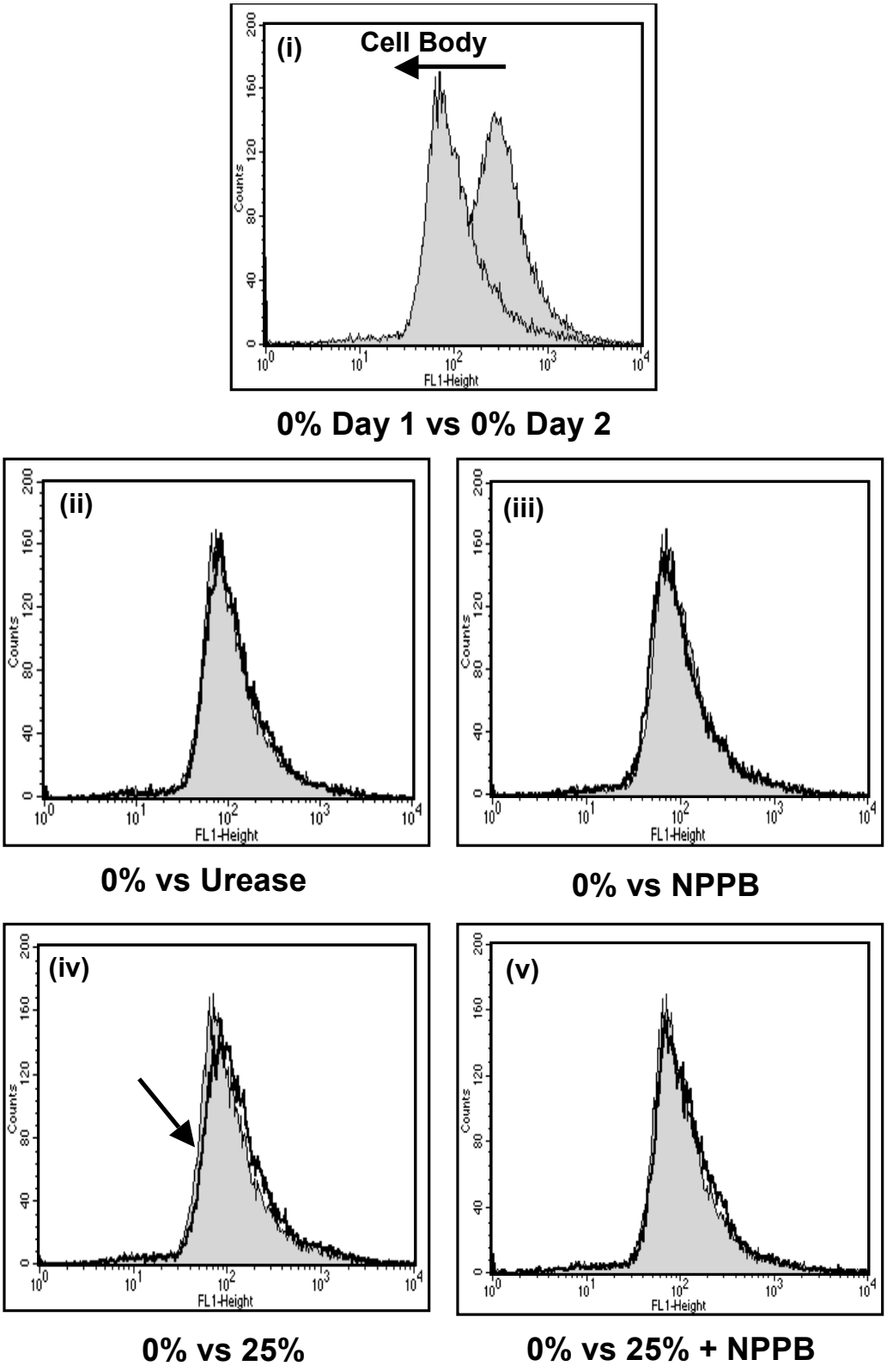


Fig. 4.2.8a: Effect of NPPB on *HPCM*-induced changes in BAEC proliferation. Following BAEC exposure to 25% HPCM for 48 h in the absence and presence of 100 mM NPPB, cells were monitored for proliferation by FACS Analysis. (i) Arrow shows shift in cell body after 2 days (ii) 0 vs. Urease (iii) 0 vs. NPPB (iv) 0 vs. 25%, arrow indicates shift in cell body (v) 0 vs. NPPB. Grey shaded area represents untreated control cells whilst black line represents *HPCM* treated cells. Curves are representative.

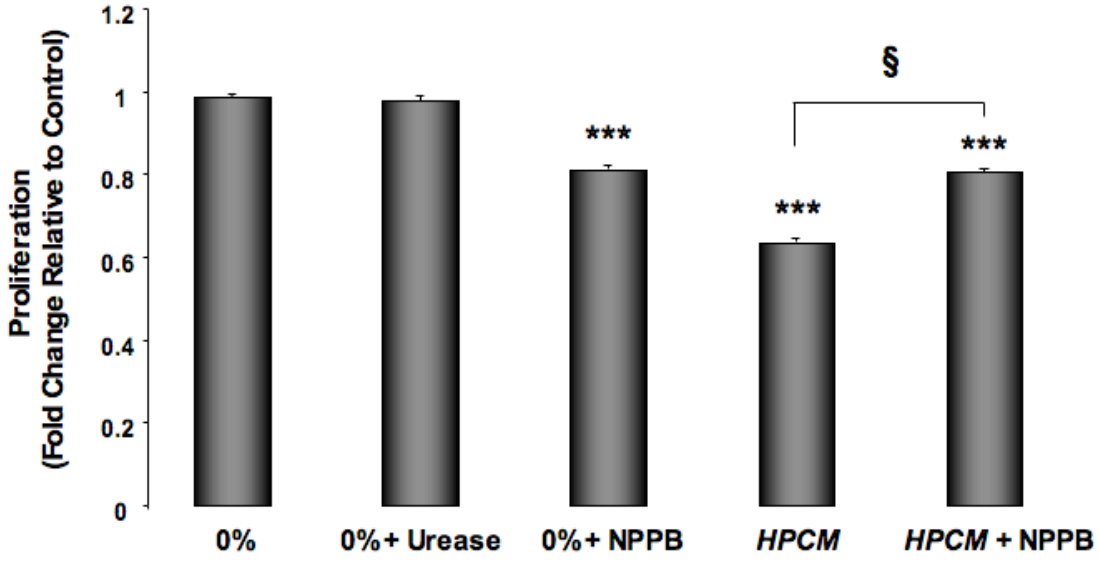


Fig. 4.2.8b: Effect of NPPB on *HPCM*-induced changes in BAEC proliferation. Following BAEC exposure to 25% HPCM for 24 h in the absence and presence of 100 mM NPPB, cells were monitored for proliferation by cell counts. Histogram represents fold change relative to control and is averaged from three independent experiments. ±SEM. ***$P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus 25% *HPCM*.

4.2.9 NPPB blocks the *HPCM*-mediated decrease in BAEC barrier function.

Following exposure of sheared BAECs to 25% *HPCM* for 24 h in the absence and presence of NPPB, cells were immunocytochemically monitored for ZO-1 localization, as described previously. Consistent with an intact endothelial barrier, ZO-1 immunoreactivity exhibited a continuous, well-defined pattern of localization along the cell-cell border under untreated conditions in the absence or presence of NPPB, Urease and *E. coli* conditioned media (Fig. 4.2.9 i-iii, vii). Following 25% *HPCM* treatment however, ZO-1 membrane localization became highly discontinuous and jagged along the cell-cell border, an effect that was prevented by treatment with NPPB (Fig. 4.2.9 vi).

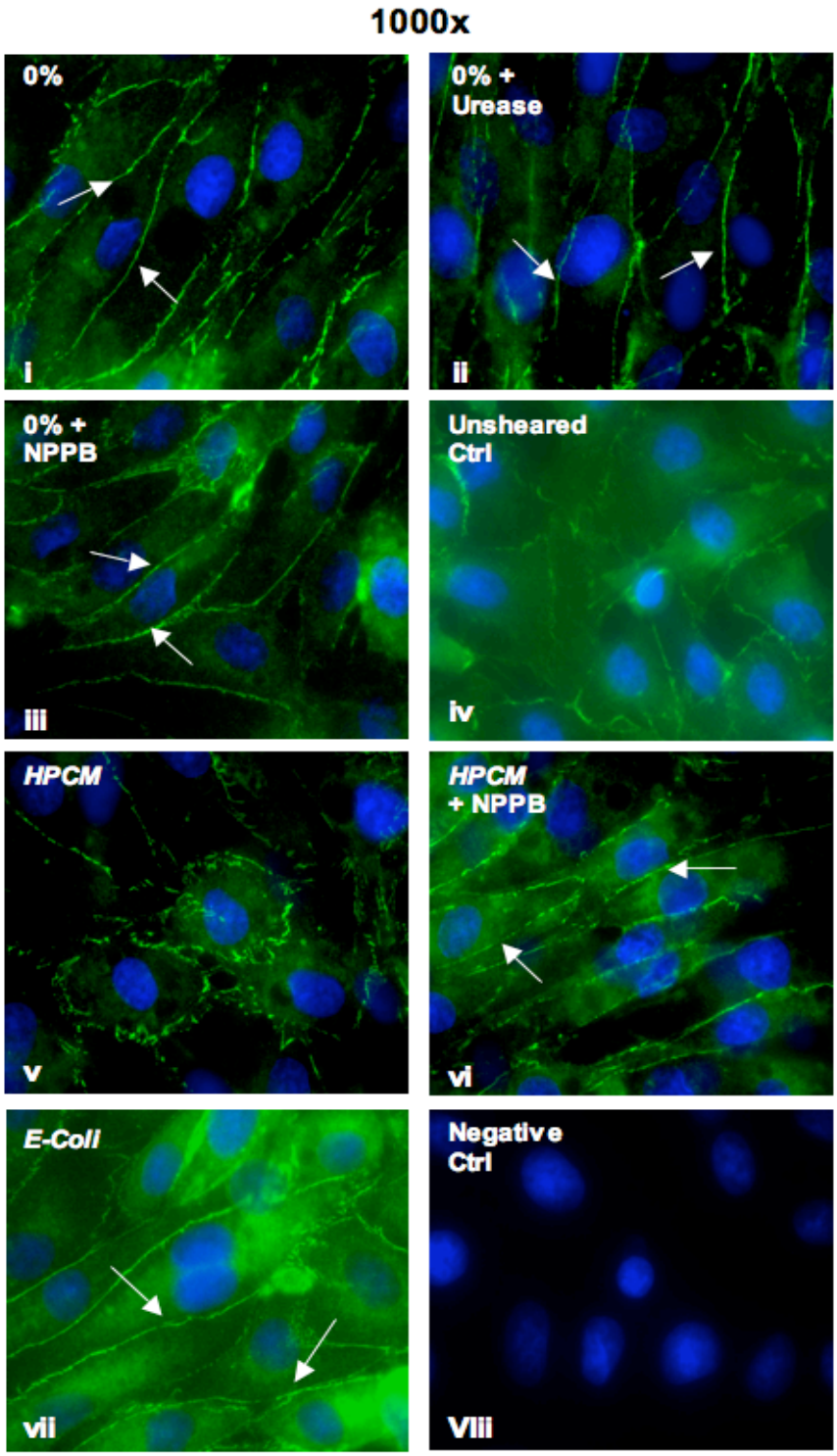


Fig. 4.2.9: Effect of NPPB on HPCM-induced changes in BAEC ZO-1 membrane localization. Following exposure of cyclically strained BAECs to 25% HPCM for 24 h in the absence and presence of 100 mM NPPB and Urease (10 μg/mL), cells were immunocytochemically monitored for ZO-1 localization. White arrows indicate plasma membrane localization of ZO-1. (i) 0% (ii) 0% + Urease (iii) 0% + NPPB (iv) Unsheared Control (v) 25% *HPCM* (vi) 25% *HPCM* + NPPB (vii) *E. coli* (viii) Negative control with DAPI stained nuclei. Images are representative.

4.2.10 VacA$^-$ *HPCM* does not induce vacuole formation in BAECs.

Following BAEC exposure to *HPCM* (100%) from VacA$^+$ and VacA$^-$ (VacA negative strain produced from the parental 60190 VacA$^+$ strain through gene deletion) for 24 h, cell vacuolation was examined by vacuolation assay. BAECs incubated in either VacA$^-$ *HPCM* or *E. coli* conditioned media showed no vacuolation (Fig. 4.2.10 ii, iv). Cells incubated with VacA$^+$ *HPCM* showed extensive vacuolation (Fig. 4.2.10 iii).

4.2.11 VacA$^-$ *HPCM* has no effect on BAEC migration or tube formation.

Following BAEC exposure to *HPCM* (25%) from VacA$^+$ and VacA$^-$ strains for 24 h, cells were monitored for migration and tube formation. BAEC migration was reduced to 0.70±2.26 fold of control following incubation with VacA$^+$ *HPCM*, whilst VacA$^-$ *HPCM* did not alter migration in comparison to untreated control (Fig. 4.2.13 a). Similarly, BAEC tube formation was reduced to 0.41±0.01 of control with VacA$^+$ *HPCM,* whilst VacA$^-$ *HPCM* had no effect (Fig. 4.2.13 b). In both experiments, *E. coli* conditioned media was included as a gram-negative bacterial control, and had no effect.

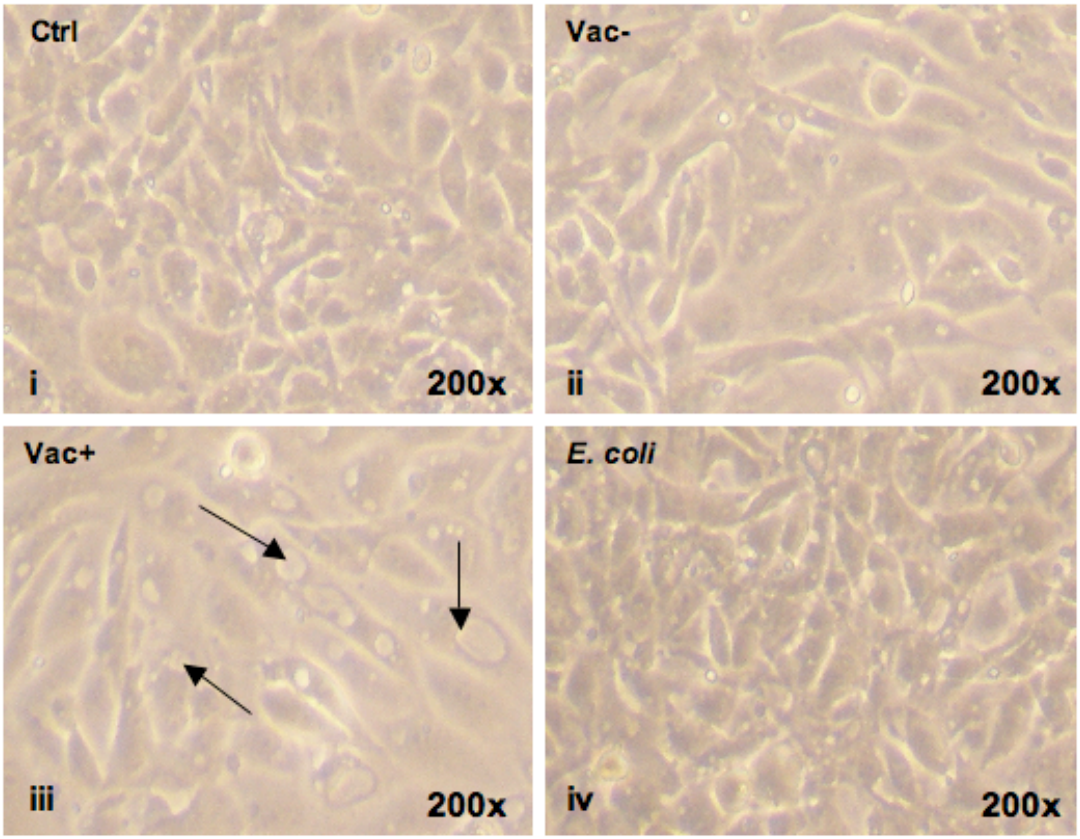


Fig. 4.2.10: Effect of different *HPCM* strains on BAEC vacuolation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and Vac^- 60190 strains) and *E. coli* conditioned media for 24 h, cells were examined by vacuolation assay. Black arrows indicate vacuoles. (i) Ctrl no conditioned media (ii) Vac^- *HPCM* (iii) Vac^+ *HPCM* (iv) *E. coli* conditioned media. Images are representative.

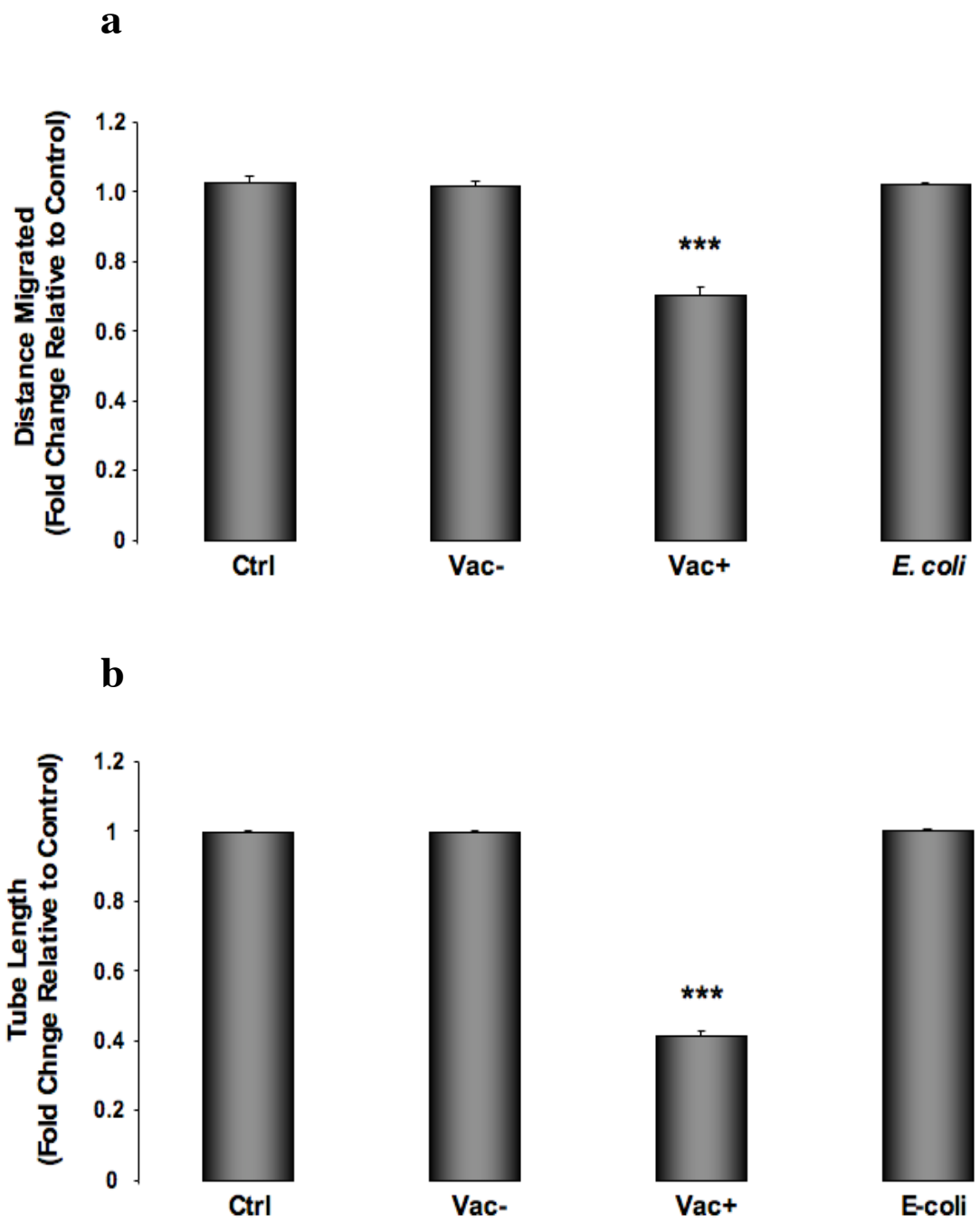


Fig. 4.2.11: Effect of different *HPCM* strains on BAEC tube formation and migration Following BAEC exposure to 25% HPCM ($VacA^+$ and Vac^- strains) for 24 h, cells were monitored for (a) Tube formation and (b) Migration. Histograms are averaged from three independent experiments ±SEM. *E. coli*- conditioned media included as gram-negative bacterial control. ***$P \leq 0.005$ versus control.

4.2.12 VacA$^-$ *HPCM* has no effect on BAEC proliferation.

Following BAEC exposure to *HPCM* (25%) from VacA$^+$ and VacA$^-$ strains for 4 days, cell proliferation was examined by FACS analysis and cell counts. FACS analysis showed a decrease in proliferation of BAECs incubated with VacA$^+$ *HPCM*, while VacA$^-$ *HPCM* had no effect (Fig. 4.2.12a).

Similarly, cell counts revealed proliferation was reduced to 0.65±0.01 fold of control following incubation with VacA$^+$ *HPCM*, however VacA$^-$ *HPCM* had no effect (Fig. 4.2.12b). In both experiments *E. coli*- conditioned media was included as a gram-negative bacterial control, and had no effect relative to untreated control.

4.2.13 VacA$^-$ *HPCM* has no effect on BAEC barrier function.

Following exposure of sheared BAECs to *HPCM* (25%) from VacA$^+$ and VacA$^-$ strains, cells were immunocytochemically monitored for ZO-1 localization. ZO-1 immunoreactivity exhibited a continuous, well-defined pattern of localization along the cell-cell border under control and VacA$^-$ conditions (Fig. 4.2.13 i-ii). Under VacA$^+$ conditions however, ZO-1 membrane localization became highly discontinuous and jagged along the cell-cell border (Fig. 4.2.13 iii).

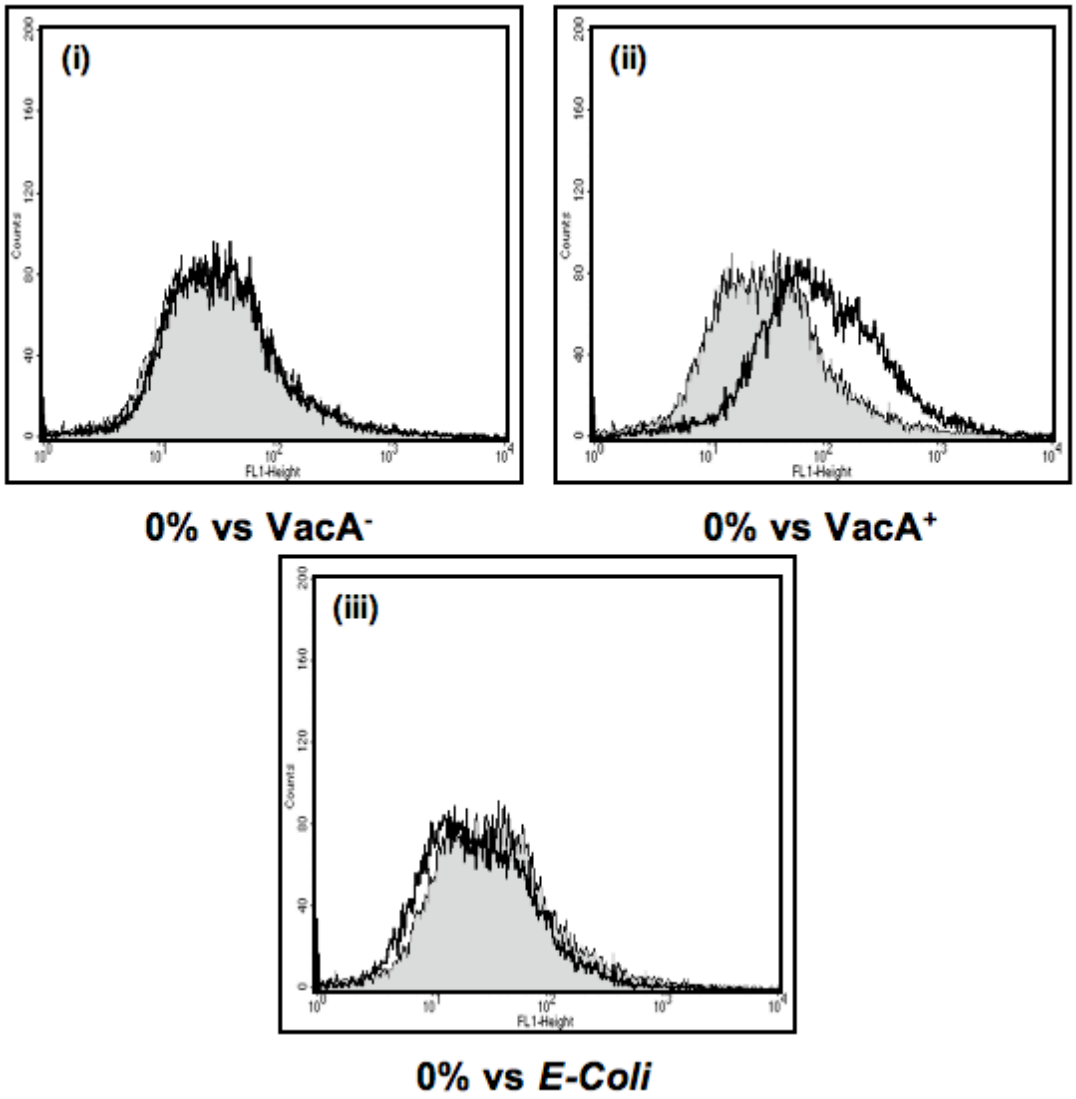


Fig. 4.2.12a: Effect of different *HPCM* strains on BAEC proliferation. Following BAEC exposure to 25% HPCM ($VacA^+$ and $VacA^-$ strains) for 24 h, cells were monitored for proliferation by FACS analysis. Grey shaded area represents untreated control cells whilst black line represents conditioned media treated cells. *E. coli*- conditioned media included as gram-negative bacterial control. (i) 0% vs. $VacA^-$ (ii) 0% vs. $VacA^+$ (iii) 0% vs. *E. coli* conditioned media. Curves are representative.

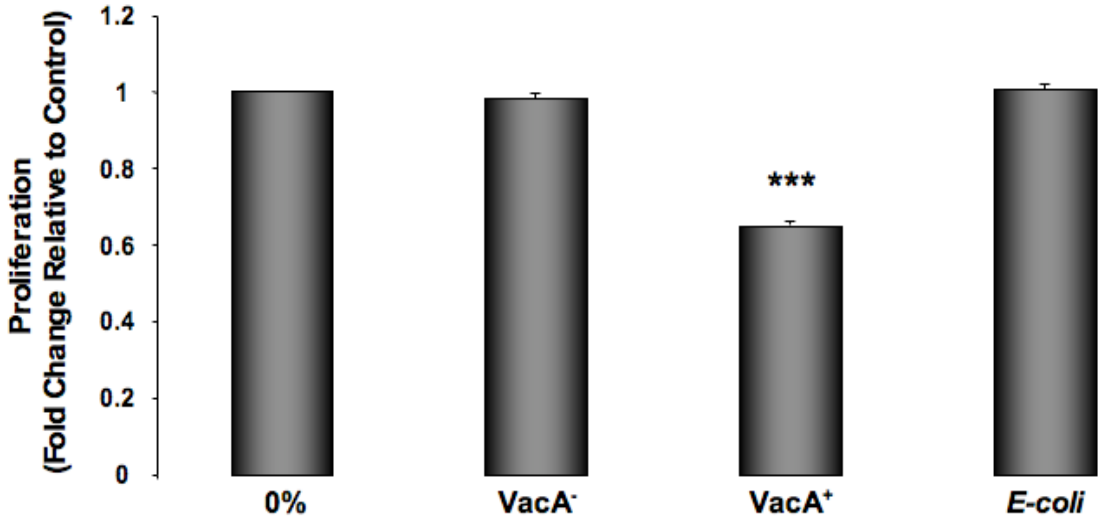


Fig. 4.2.12b: Effect of different *HPCM* strains on BAEC Proliferation. Following BAEC exposure to 25% HPCM ($VacA^+$ and $VacA^-$ strains) for 24 h, cells were monitored for proliferation by cell counts. *E. coli*- conditioned media included as gram-negative bacterial control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control.

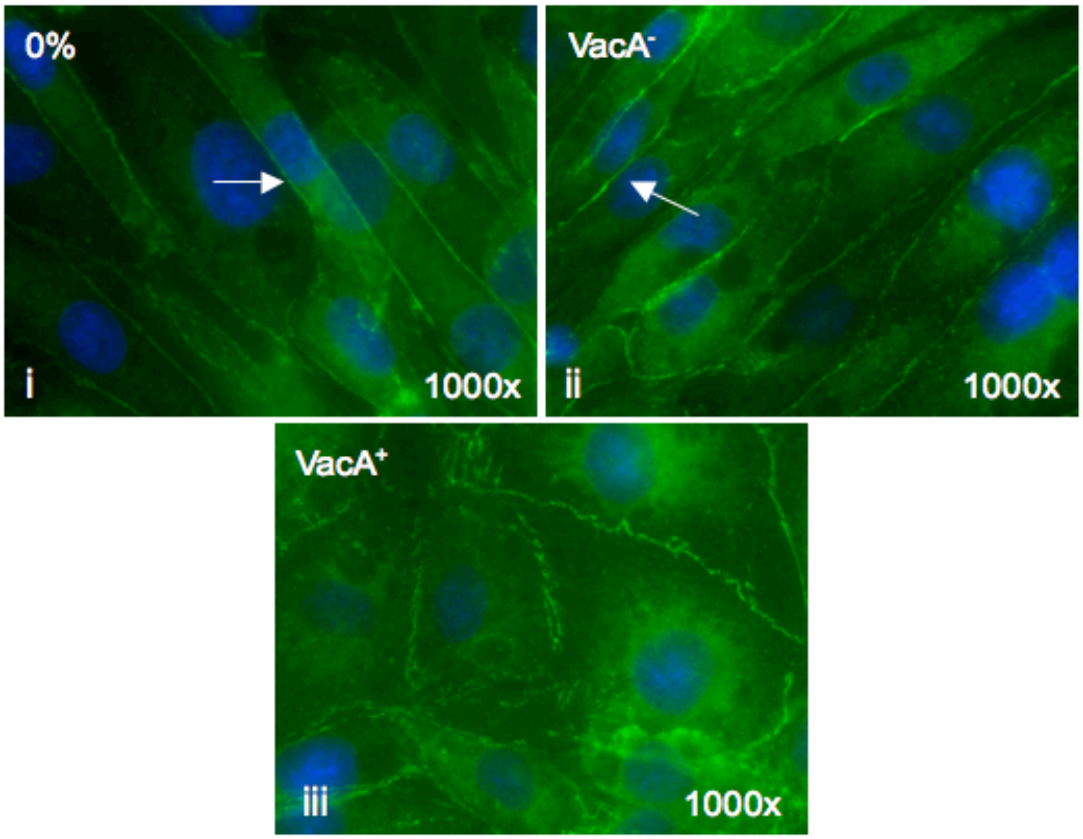


Fig. 4.2.13: Effect of different *HPCM* strains on BAEC ZO-1 membrane localization. Following exposure of cyclically strained BAECs to 25% HPCM ($VacA^+$ and Vac^- strains) for 24 h cells were immunocytochemically monitored for ZO-1 localization. White arrows indicate plasma membrane localization of ZO-1. DAPI-stained nuclei are clearly visible. (i) 0% *HPCM* (ii) 25% $VacA^-$ *HPCM* (iii) 25% $VacA^-$ *HPCM*. Images are representative.

4.2.14 VacA$^-$ *HPCM* has no effect on BAEC permeability

Following exposure of sheared BAECs to 25% *HPCM* (cells are initially sheared to increase permeability so that change is more discernible) from VacA$^+$ and VacA$^-$ strains, VacA$^-$ *HPCM* caused no increase in permeability over control. BAECs incubated with VacA$^+$ *HPCM* increased permeability 1.127 fold. *E. coli*- conditioned media was included as a gram-negative bacterial control and had no effect (Fig. 4.2.14).

4.2.15 VacA$^-$ *HPCM* has no effect on BAEC nitrite production.

Following exposure of BAECs to 25% *HPCM* from VacA$^+$ and VacA$^-$ strains, changes in sodium nitrite production were examined by DAN assay. Incubation of BAECs with VacA$^+$ *HPCM* reduced sodium nitrite levels to 0.46±0.02 fold of control. *E. coli*- conditioned media had no effect (Fig. 4.2.15).

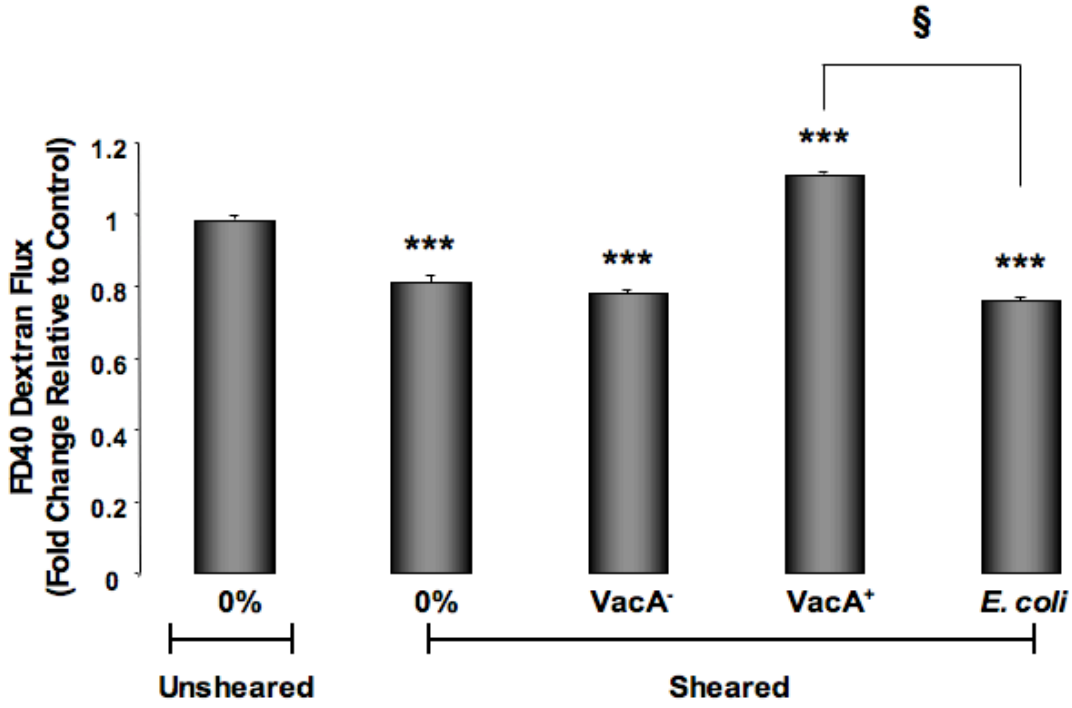


Fig. 4.2.14: Effect of different *HPCM* strains on BAEC. Following exposure of sheared (10 dyne/cm^2) BAECs to 25% HPCM (VacA$^+$ and VacA$^-$ strains) for 24 h cells were cells were monitored for permeability. *E. coli*- conditioned media was included as gram-negative bacterial control. Histograms represent fold change relative to unsheared control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus VacA$^+$.

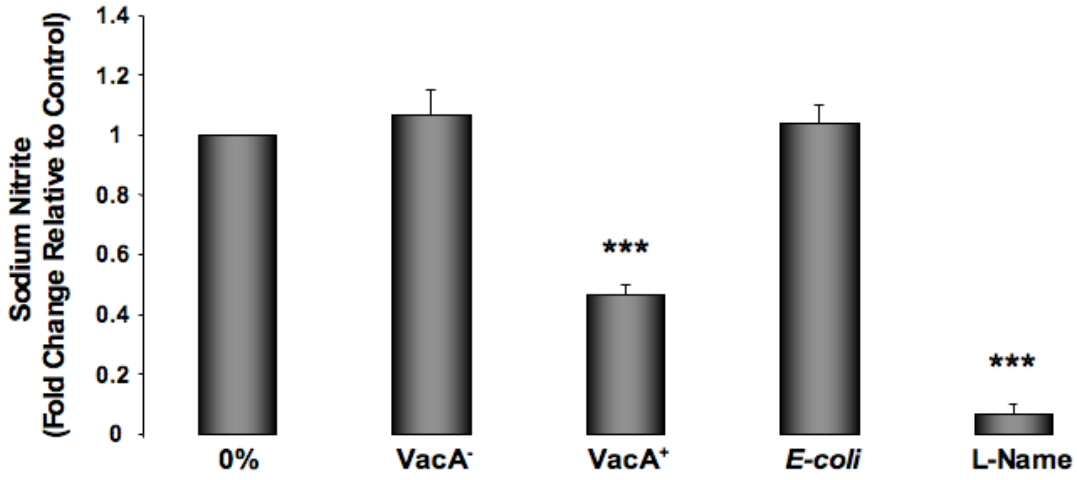


Fig. 4.2.15: Effect of different *HPCM* strains on BAEC Permeability and Nitrite production Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) for 24 h, cells were monitored for Nitrite production. *E. coli*- conditioned media included as positive control, L-Name included as negative control. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control.

4.2.16 SNAP recovers the VacA$^+$ *HPCM*-mediated decrease in BAEC migration and tube formation.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$) for 24 h in the absence and presence of SNAP, cells were monitored for migration and tube formation. Exposure of cells to SNAP (1 μM) had no effect on migration relative to control, whilst VacA$^+$ *HPCM* reduced migration to 0.75±0.011 fold of control. Exposure of BAECs to VacA$^+$ *HPCM* in the presence of SNAP completely prevented the *HPCM*-dependent reduction in BAEC migration (Fig. 4.2.16 a). Furthermore, exposure of cells to VacA$^+$ *HPCM* also reduced tube formation to 0.44±0.02 fold of control, an effect that was completely prevented by treatment with SNAP (5 μM) (Fig. 4.2.16 b). VacA$^-$ *HPCM* was included in both experiments and had no effect on BAEC migration or tube formation.

4.2.17 SNAP recovers the VacA$^+$ *HPCM*-mediated decrease in BAEC proliferation- FACS Analysis.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$) in the absence and presence of SNAP (0.25 μM), cells were monitored for proliferation over 4 days by FACS Analysis. As previously shown, 25% VacA+ *HPCM* reduced proliferation relative to control, however inclusion of SNAP in VacA+ *HPCM* completely prevented the effect of *HPCM* on BAEC proliferation. VacA$^-$ *HPCM* had no effect on proliferation (Fig. 4.2.17).

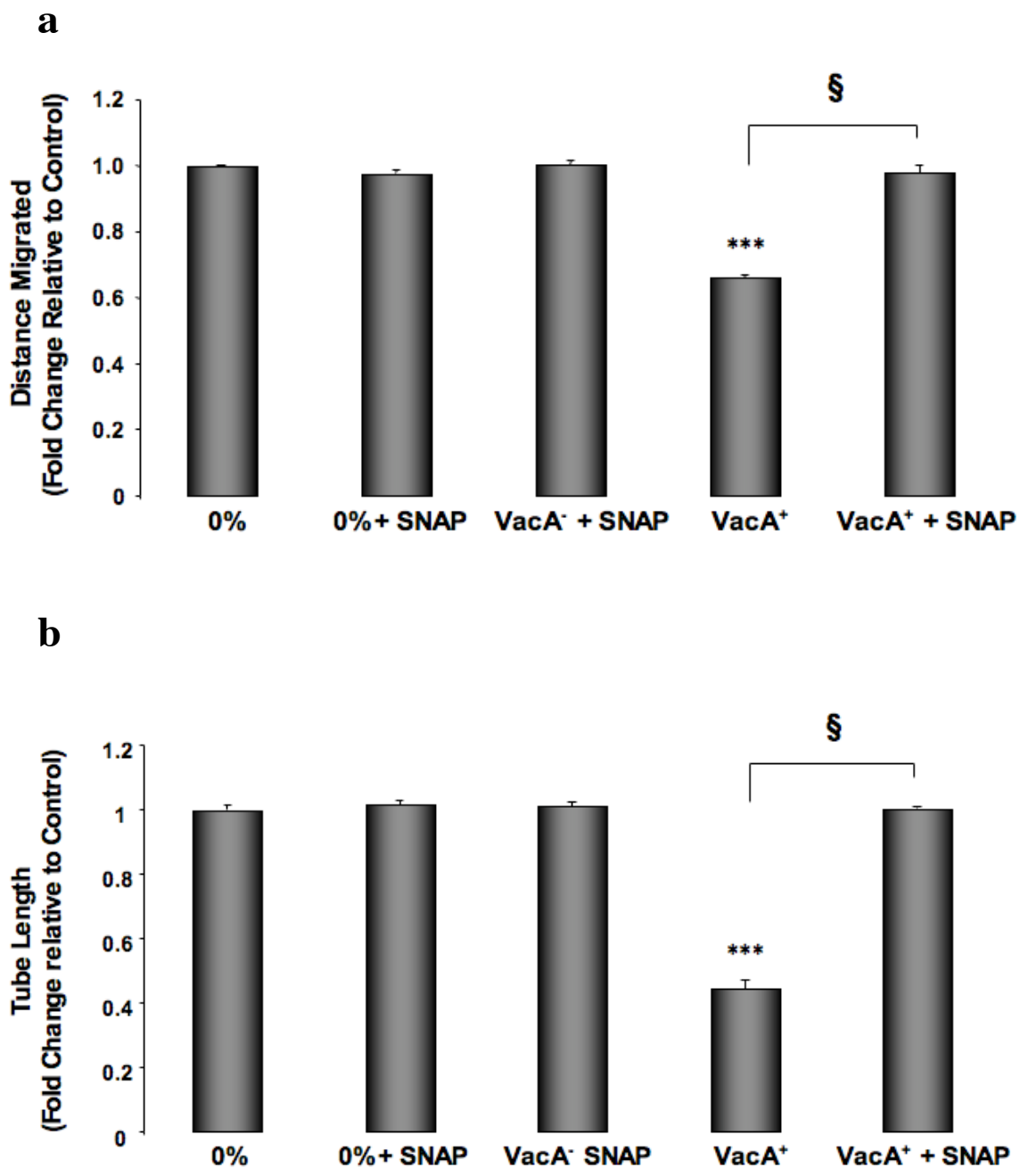


Fig. 4.2.16 : Effect of SNAP on *HPCM*-induced changes in BAEC Migration and Tube Formation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) in the absence and presence of SNAP for 24 h, cells were monitored for (a) Migration and (b) Tube formation- by collagen based tube formation assay. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus $VacA^+$.

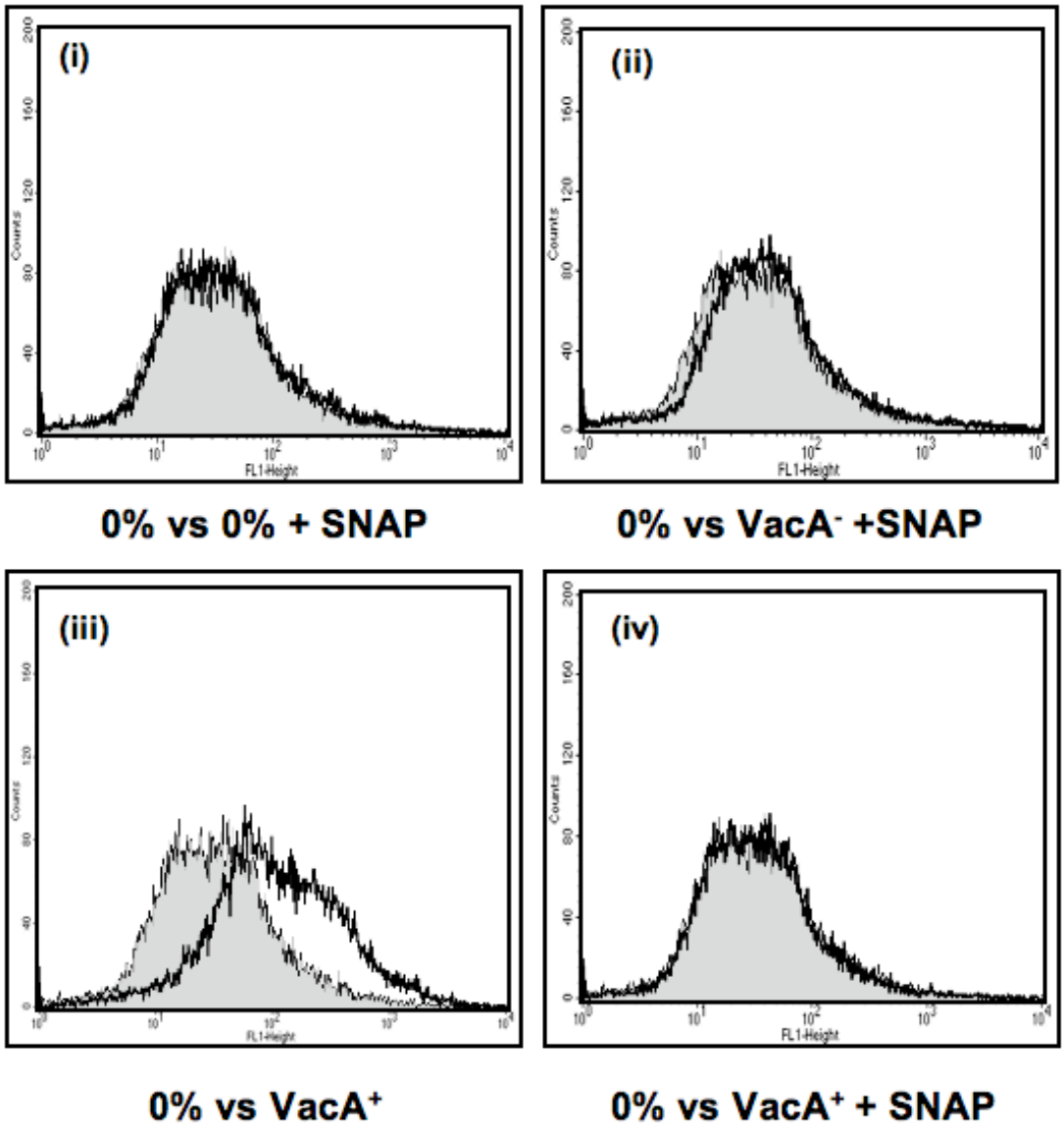


Fig. 4.2.17: Effect of SNAP on *HPCM*-induced changes in BAEC proliferation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) for 24 h in the absence and presence of SNAP, cells were monitored for proliferation by FACS Analysis. Grey shaded area represents untreated control cells whilst black line represents HPCM and inhibitor treated cells. (i) 0% vs. 0% + SNAP (ii) 0% vs. $VacA^-$ + SNAP (iii) 0% vs. $VacA^+$ (iv) 0% vs. $VacA^+$ + SNAP. Curves are representative.

4.2.18 SNAP recovers the VacA$^+$ *HPCM*-mediated decrease in BAEC proliferation- cell counts.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$) in the absence and presence of SNAP (0.25 μM), cells were monitored for proliferation over 4 days by cell counts. Exposure of cells to 25% VacA$^+$ *HPCM* reduced proliferation to 0.56±0.01 fold of control an effect which was completely prevented by the inclusion of SNAP. VacA$^-$ *HPCM* had no effect on proliferation (Fig 4.2.18).

4.2.19 SNAP recovers the VacA$^+$ *HPCM*-mediated decrease in BAEC barrier function.

Following exposure of sheared BAECs to 25% *HPCM* (VacA$^+$ and VacA$^-$) for 24 h in the absence and presence of SNAP (1 μM), cells were immunocytochemically monitored for ZO-1 localization. Consistent with an intact endothelial barrier, ZO-1 immunoreactivity exhibited a continuous, well-defined pattern of localization along the cell-cell border under untreated conditions and in the absence or presence of SNAP and VacA$^-$ *HPCM* (Fig. 4.2.19 i-iii, v). Following VacA$^+$ *HPCM* treatment however, ZO-1 membrane localization became highly discontinuous and jagged along the cell-cell border, an effect that was substantially prevented by treatment with SNAP (Fig. 4.2.19 v).

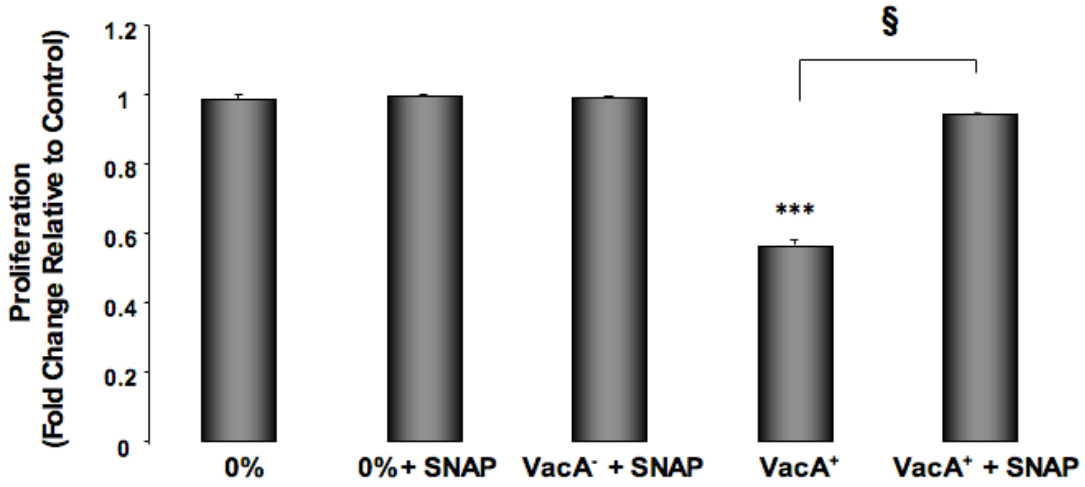


Fig. 4.2.18: Effect of SNAP on *HPCM*-induced changes in BAEC proliferation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) for 24 h in the absence and presence of SNAP, cells were monitored for proliferation by cell counts. Histograms represent fold change relative to control and are averaged from three independent experiments ±SEM. $^{***}P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus $VacA^+$.

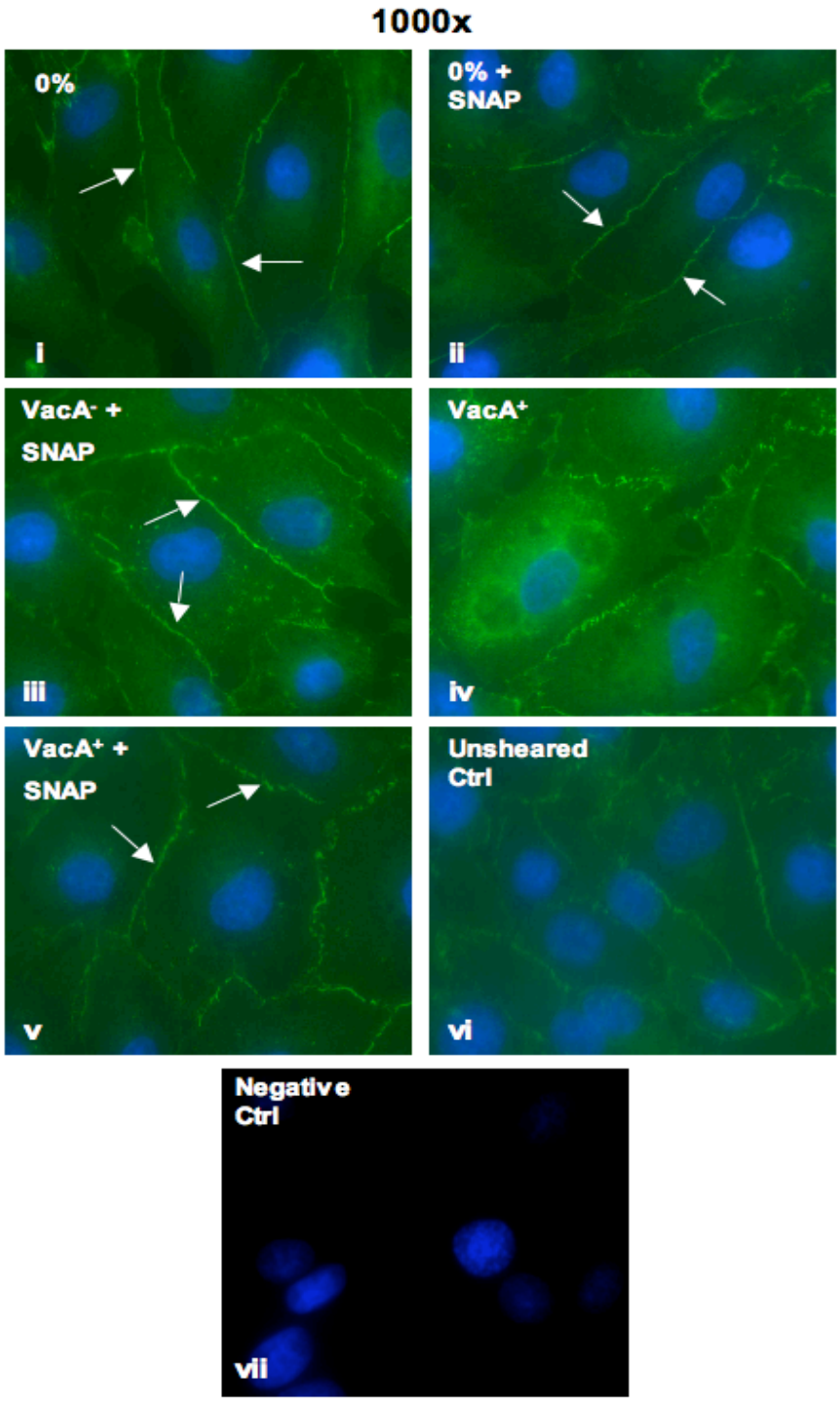


Fig. 4.2.19: Effect of SNAP on HPCM-induced changes in BAEC ZO-1 membrane localization. Following exposure of sheared BAECs to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) for 24 h in the absence and presence of SNAP, cells were immunocytochemically monitored for ZO-1 localization. White arrows indicate plasma membrane localization of ZO-1. DAPI-stained nuclei are clearly visible. (i) 0% (ii) 0% + SNAP (iii) $VacA^-$ + SNAP (iv) $VacA^+$ (v) $VacA^+$ + SNAP (vi) Unsheared control (vii) Negative control. Images are representative.

4.2.20 SNAP recovers the VacA$^+$ *HPCM*-mediated increase in BAEC permeability.

Following exposure of sheared BAECs to 25% *HPCM* (VacA$^+$ and VacA$^-$ strains) for 24 h, cell permeability was assessed by FD40 Dextran permeability assay. Exposure of cells to VacA$^+$ *HPCM* increased permeability by 3.69±0.11 fold relative to sheared controls, an effect which could be prevented by the inclusion of SNAP (1 μM) (Fig 4.2.20).

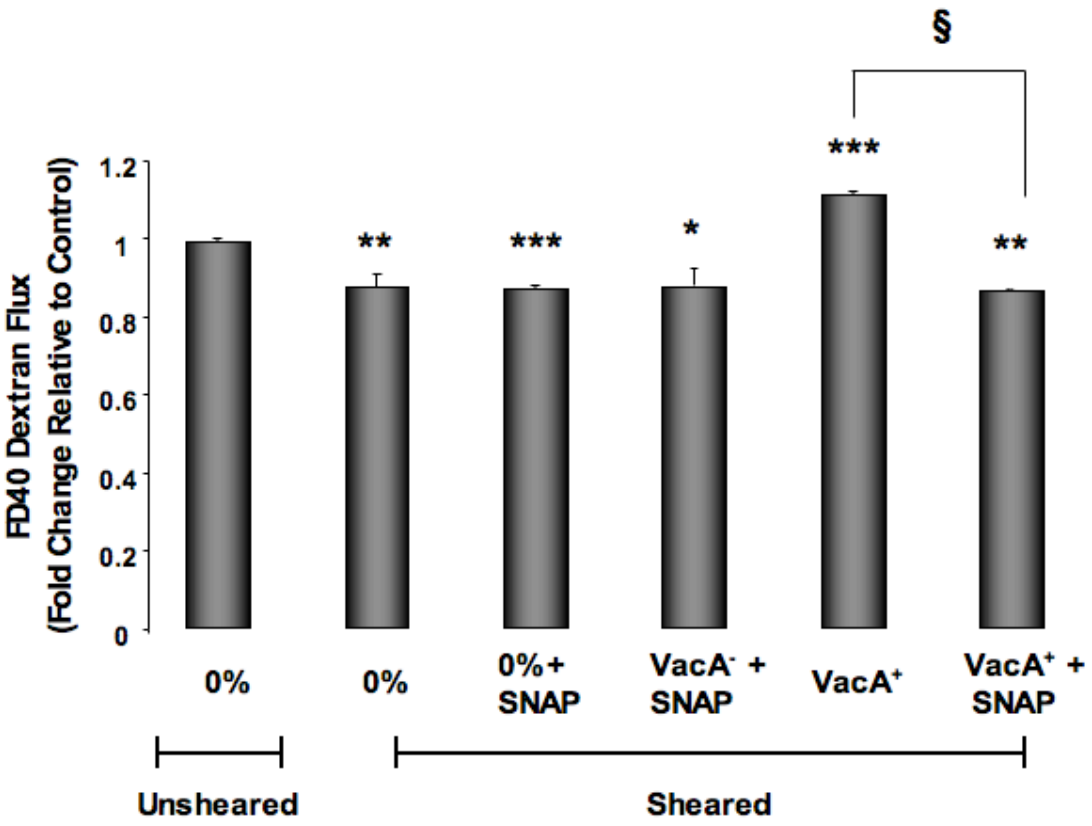


Fig. 4.2.20: Effect of SNAP on *HPCM*-induced changes in BAEC permeability. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) for 24 h in the absence and presence of SNAP, cells were monitored for for permeability. Histograms represent fold change relative to unsheared control and are averaged from three independent experiments ±SEM. $^*P \leq 0.5$ versus control. $^{**}P \leq 0.05$ versus control. $^{***}P \leq 0.005$ versus control. $^{\S}P \leq 0.005$ versus $VacA^+$.

4.3 Discussion

The endothelial cells lining the vascular system form an interface between bloodflow and underlying smooth mucle cell layers, providing a highly effective fluid and solute barrier. The endothelium is constantly exposed to any foreign matter that enters the blood stream, and its continued homeostasis is vital in preventing the development cardiovascular disease. Dysfunction of the endothelial layer is considered an early indicator in the onset of vascular occlusive pathologies such as atherosclerosis and intimal hyperplasia. While numerous traditional risk factors such as obesity, smoking and lack of exercise are associated with endothelial dysfunction, leading to atherosclerotic plaque formation, some non-traditional factors have also emerged such as elevated C-reactive protein levels (Schwedler *et al.*, 2007), homocysteine levels (Caramia and Belardinelli, 2006), and bacterial infection. The latter is the focus of much experimental research, with many groups reporting associations for (Hoffmeister *et al.*, 2001, Majka *et al.*, 2002, Pieniazek *et al.*, 1999, Zito *et al.*, 1999) and against (Koenig *et al.*, 1999, Pellicano *et al.*, 2002, Whincup *et al.*, 2000) the relationship. With respect to bacterial infection, no group have, to our knowledge, focused on cells of aortic or coronary artery origin. In addition, as it appears likely that the whole bacteria would be destroyed if it were to vacate the stomach, the role of bacterial secretions in cell function must also be explored. To address these matters, we have examined the effect of *HPCM* on BAEC function.

Initial investigations clearly demonstrated that exposure of BAECs to *HPCM* for 24 h dose-dependently induced vacuolation, significant re-distribution of actin bundles, and an apoptotic phenotype. Concomitantly, BAEC proliferation was

inhibited in a dose-dependent manner at sub-apoptotic concentrations (0-25%). These results mirror previous studies with *H. pylori* extracts on microvascular endothelial cells and HUVECs (Kalia *et al.*, 2001, Kurosawa *et al.*, 2002, Pearce *et al.*, 2004, Jenkinson *et al.*, 2002). Further experiments also revealed a dose-dependent reduction in BAEC tube formation and migration following 24 h incubation with *HPCM* (0-25%). Kalia *et al.,* and others have reported similar results in microvascular endothelial cells (Jenkinson *et al.*, 2002, Kalia *et al.*, 2001), while opposing studies by Pearce *et al.*, showed no effect of *H. pylori* extracts on HDMvEC or HUVEC migration (Pearce *et al.*, 2004).

As previously mentioned (section 3.3), immunoreactivity of zonnula occludens-1 (ZO-1) has been successfully employed as an index of barrier integrity by our group and others (Bendfeldt et al., 2007, Colgan et al., 2007b). In our studies, ZO-1 localization was well defined and continuous along cell-cell junctions in control cells. However, following exposure of BAECs to 25% *HPCM* for 24 h, localization became jagged and discontinuous. These results are consistent with *H. pylori* disruption of tight junction assembly and barrier integrity and concur with the previous studies of Brisslert *et al.*, which indicated that *HPCM* significantly increased recruitment and transendothelial migration of neutrophils in HUVECs (Brisslert *et al.*, 2005).

Taken together, these results are indicative of a role for *H. pylori* secretions in an inflammatory process such as that associated with vascularocclusive remodelling and atherosclerosis. Loss of barrier function and dysregulation of endothelial cell function are central to the initiation and progression of

atherosclerotic lesion development (Simionescu, 2007). A number of *H. pylori* virulence factors have been described with the potential to cause these effects. The role of OMP's from the bacterium in this regard has already been discussed (section 3.3). Urease secreted by *H. pylori* is known to induce inflammatory responses (Enarsson *et al.*, 2005) and upregulate iNOS expression (Fu *et al.*, 1999). Both VacA and CagA have been implicated in altering host cell and barrier function (Amieva *et al.*, 2003, Willhite and Blanke, 2004). Along with these virulence factors, there are numerous other secretions from *H. pylori* (sections 1.4.3.4- 1.4.3.6) whose plausible contribution to atherosclerosis have not been explored. However, in our studies bacterial conditioning of media proceeded for 24 h only. Studies by Schraw *et al.,* have determined the secretion profile of the bacteria and show during this time period that VacA is the only major protein produced. Urease and Hsp B are also early secretions but occur after the 24 h timepoint (Schraw *et al.*, 1999). Hence we examined the effect of VacA and Urease on BAEC cell function.

The enzyme Urease accounts for almost 10% of total *H. pylori* protein, and functions in converting urea in the stomach to ammonia, thereby neutralizing the acid surrounding the bacterium and facilitating gastric colonization. Apart from its role in inflammation (mentioned above), Fan *et al.,* have shown a urease-dependent induction of apoptosis in gastric epithelial cells (Fan et al., 2000). These effects suggest the ability of urease to influence the progression of an atherosclerotic phenotype. For our studies we examined BAECs following treatment with unconditioned control media containing 10 μg/mL jack bean urease (known to display many of the same properties as *H. pylori* urease (Olivera-Severo *et al.*,

2006)). Urease had no effect on any of the cell functions we studied, suggesting its lack of involvement in any of the *HPCM*-mediated events examined here.

As previously discussed (section 1.4.3.1), VacA is an autotransporter protein responsible for large vacuole formation in cultured mammalian cells. It functions by forming anion channels in the host cell plasma membrane, with resulting membrane depolarization (Szabo *et al*., 1999). In addition, VacA can enter cells and modulate mitochondrial permeability causing cytochrome C release (Willhite and Blanke, 2004), reduce gastric epithelial barrier function (Pelicic *et al*., 1999), inhibit T-lymphocyte proliferation and cytokine release (Gebert *et al*., 2004), and induce gastric epithelial apoptosis (Cover *et al*., 2003). Moreover, VacA- dependent regulation of the expression and function of cytoskeleton-associated Rho-GTPase family proteins has also been reported for gastric epithelial cells (Pai *et al*., 2000). In contrast, little information is available for the effects of *H. pylori*-derived VacA on endothelial cells. Kurosawa *et al*. reports that the *H. pylori*-dependent reduction in HUVEC proliferation, observed in parallel with induction of apoptosis, is seen with *both* $CagA^+/VacA^+$ double-positive and $CagA^-/VacA^-$ double-negative strains. However, this is not conclusive evidence of a role of VacA in endothelial function, as the study suggests (Kurosawa *et al*., 2002).

To further our understanding of VacA, we employed NPPB, an inhibitor known to selectively block the action of *H. pylori*-derived VacA (Szabo *et al*., 1999). Treatment of BAECs with NPPB revealed a baseline inhibitory effect on proliferation and tube formation, observations previously reported for this inhibitor in similar studies (Manolopoulos *et al*., 2000, Rouzaire-Dubois and Dubois, 1998).

Importantly, NPPB had no effect on BAEC apoptosis as measured by AO staining-data not shown. Moreover, our data clearly showed that NPPB treatment of BAECs completely prevented *HPCM*-dependent reduction in proliferation, tube formation and migration. NPPB also completely prevents *HPCM*-dependent ablation of ZO-1 membrane localization. While these findings suggest a role for VacA in these *H. pylori*-induced changes, the possibility of an alternative NPPB-inhibitable virulence factor(s) must be taken into account. For this reason our studies were extended using the $VacA^-$ mutant of our 60190 strain (60190:v1) in which the VacA genes have been disrupted by insertational mutagenesis (Cover *et al.*, 1994).

$VacA^-$ *HPCM* had no effect relative to control on BAEC vacuolation, tube formation, migration, proliferation or ZO-1 membrane localization. The effect of *HPCM* on FD-40 Dextran flux across BAEC monolayers was also examined and it was found that $VacA^+$ *HPCM* significantly increased flux, indicating increased permeability, whilst $VacA^-$ *HPCM* had no effect relative to control.

Sodium nitrite production was next examined, a marker of endothelial health and observed a $VacA^+$ *HPCM* decrease in nitrite that was not present following exposure to $VacA^-$ *HPCM*. Taken together, these results suggest a direct role for *HPCM*-derived VacA in altering endothelial properties and function, possibly in a nitrite dependent manner.

In order to extend this premise further, BAEC cell functions following incubation with *HPCM* in the absence and presence of *S*-nitroso-*N*-acetyl pencillamine (SNAP) – a nitric oxide donor, were studies. Previous studies have shown that SNAP has the ability to increase endothelial cell migration (Kawasaki *et*

al., 2003), tube formation (Balasubramaniam *et al*., 2006), proliferation (Mottola *et al*., 2005) and permeability (Breslin *et al*., 2003). For control purposes therefore, concentrations of SNAP were used that were shown by these groups not to *increase* any of these cell properties and functions.

Our studies revealed that SNAP recovered the $VacA^+$ *HPCM*-mediated decrease in BAEC migration, tube formation, proliferation and barrier function. This implies VacA decreases endothelial function in a NO-dependent manner. These results are consistent with the observations of Liuba *et al*., who observed impaired bioreactivity of endothelial NO following co-infection of apoE-knockout mice with *Chlamydia pneumoniae* and *H. pylori* (Liuba *et al*., 2003). In addition, our studies provide vital mechanistic data to support the hypothesis concerning *H. pylori's* involvement in endothelial dysfunction.

In conclusion, among the non-traditional risk factors being proposed for cardiovascular disease, and in particular atherosclerosis, is the idea of pathogenic burden. The bacterium *H. pylori* is much studied in this role, and has been suggested to contribute to the initiation and progression of an atherosclerotic plaque through its effects on endothelial function. However, vascular endothelial cell models employed thus far are quite diverse, with little or no information available on molecular mechanisms involved, or on endothelial cells of aortic or coronary artery origin. In this study, a comprehensive examination of the effects of *H. pylori* conditioned medium on a broad range of cell properties in a well characterized aortic endothelial cell model (BAEC) was made. Our findings suggest *H. pylori*-derived factors play a complex role in an inflammatory process consistent with that typically associated

with atherosclerosis. Moreover, our experiments confirm the involvement of VacA (but not urease) in these events through a reduction in nitric oxide.

Chapter 5

Modulation of HPCM-Dependent Changes in Endothelial Cell Properties by Laminar Shear Stress

5.1 Introduction

Blood flowing in the cardiovascular system exerts two main effects on the blood vessels namely cyclic strain and shear stress. Under normal physiological conditions the shear stress to which the endothelium is exposed remains a relatively constant value of 10-15 dyne/cm^2. At these flow rates endothelial cell stability and survival is promoted and cells align in the direction of flow (Osborn *et al.*, 2006). Moreover, the flow results the production of anti-thrombotic and vasodilatory factors (van Thienen *et al.*, 2006), such as prostacyclin, NO, calcium thrombomodulin and tissue plasminogen activator. This provides a non-thrombogenic surface for blood flow and regulates vessel homeostasis along with immune and inflammatory reactions, resulting in an atheroprotective effect on the vessel wall (Yoshizumi *et al.*, 2003).

Turbulent and low levels of shear stress have been shown to occur at curvatures in the blood vessel and in arterial branch ostia and bifurcations (Davies *et al.*, 1995, Lehoux and Tedgui, 2003, Traub *et al.*, 1999). Regions of perturbed shear, in conjunction with known risk factors for cardiovascular disease, can result in signal transduction events leading to endothelial dysfunction and initiation of atherosclerotic plaque formation (Chatzizisis *et al.*, 2007). These processes are mediated by the action of NO, whose attenuation is one of the earliest biochemical changes preceding endothelial dysfunction.

Increasing evidence supports a role for *H. pylori* as a pro-atherogenic risk factor (Ando *et al.*, 2006, Grabczewska *et al.*, 2006, Kowalski *et al.*, 2002). Moreover, our current findings point to a role for the bacterium in endothelial dysfunction, with VacA-dependent attenuation of the NO pathway apparently central

to the process. However, to our knowledge, no group has examined the effects of *H. pylori*- derived VacA on cells exposed to high and low levels of shear, where an atherosclerotic phenotype typically predominates. Since laminar shear *in vivo* results in an atheroprotective effect, we hypothesize that exposure of endothelial cells to physiological levels of laminar shear *in vitro* would abrogate the $VacA^{+}$- dependent atherogenic effects on cell properties.

In this chapter, the effects of HPCM on endothelial cell properties and function under shear conditions was examined.

5.2 Results

5.2.1 Laminar Shear alters BAEC morphology and cytoskeletal formation.

Following BAEC exposure to laminar shear (10 dyne/cm^2) for 24 h, cell morphology and cytoskeletal formation were examined by phase-contrast microscopy, and immunocytochemically by F-actin staining with rhodamine-phalloidin. Laminar shear resulted in the realignment of BAECs in the direction of shear, as indicated by white arrow (Fig. 5.2.1 a ii), while F-actin staining revealed significant redistribution of actin bundles when compared to unsheared control (Fig. 5.2.1 b i, ii).

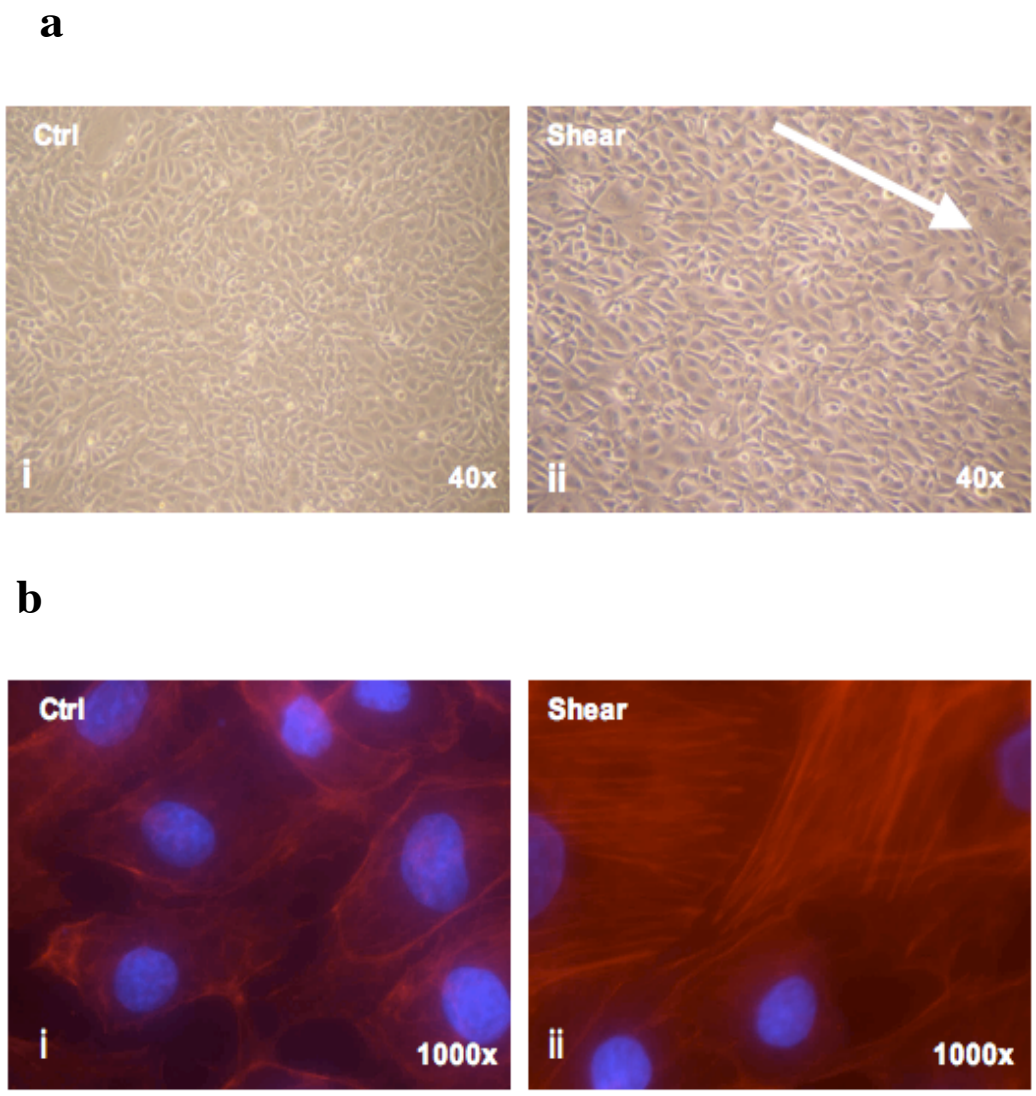


Fig. 5.2.1: Effect of Shear on BAEC morphology and cytoskeleton. Following BAEC exposure to shear (10 dyne/cm^2) for 24 h, cells were examined for (a) Morphology (b) Cytoskeletal rearrangement. White arrow indicates direction of shear. (a) Phase contrast (i) Ctrl (Unsheared) (ii) Sheared (b) Rhodamine-Phalloidin (i) Ctrl (Unsheared) (ii) Sheared. Images are representative.

5.2.2 Laminar shear restores the VacA$^+$ *HPCM*-mediated decrease in BAEC migration and tube formation.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$) under shear (0- 10 dynes/cm^2) for 24 h, cells were monitored for migration and tube formation. Shearing at 10 dyne/cm^2 increased baseline levels of both migration and tube formation. VacA$^+$ *HPCM* decreased migration by 0.70±2.26 and 0.72±2.97 fold of 0% control at 0 and 1 dyne/cm^2, respectively, although shearing restored migration to baseline control levels seen at 10 dyne/cm^2. VacA$^-$ *HPCM* and *E. coli*-conditioned media had no effects relative to 0% controls (Fig. 5.2.2 a).

Similarly, VacA$^+$ *HPCM* decreased tube formation by 0.41±0.01 and 0.42±0.001 fold of 0% control at 0 and 1 dyne/cm^2, respectively, and shearing restored migration to baseline control levels seen at 10 dyne/cm^2. VacA$^-$ *HPCM* and *E. coli*- conditioned media had no effects relative to 0% controls (Fig. 5.2.2 b).

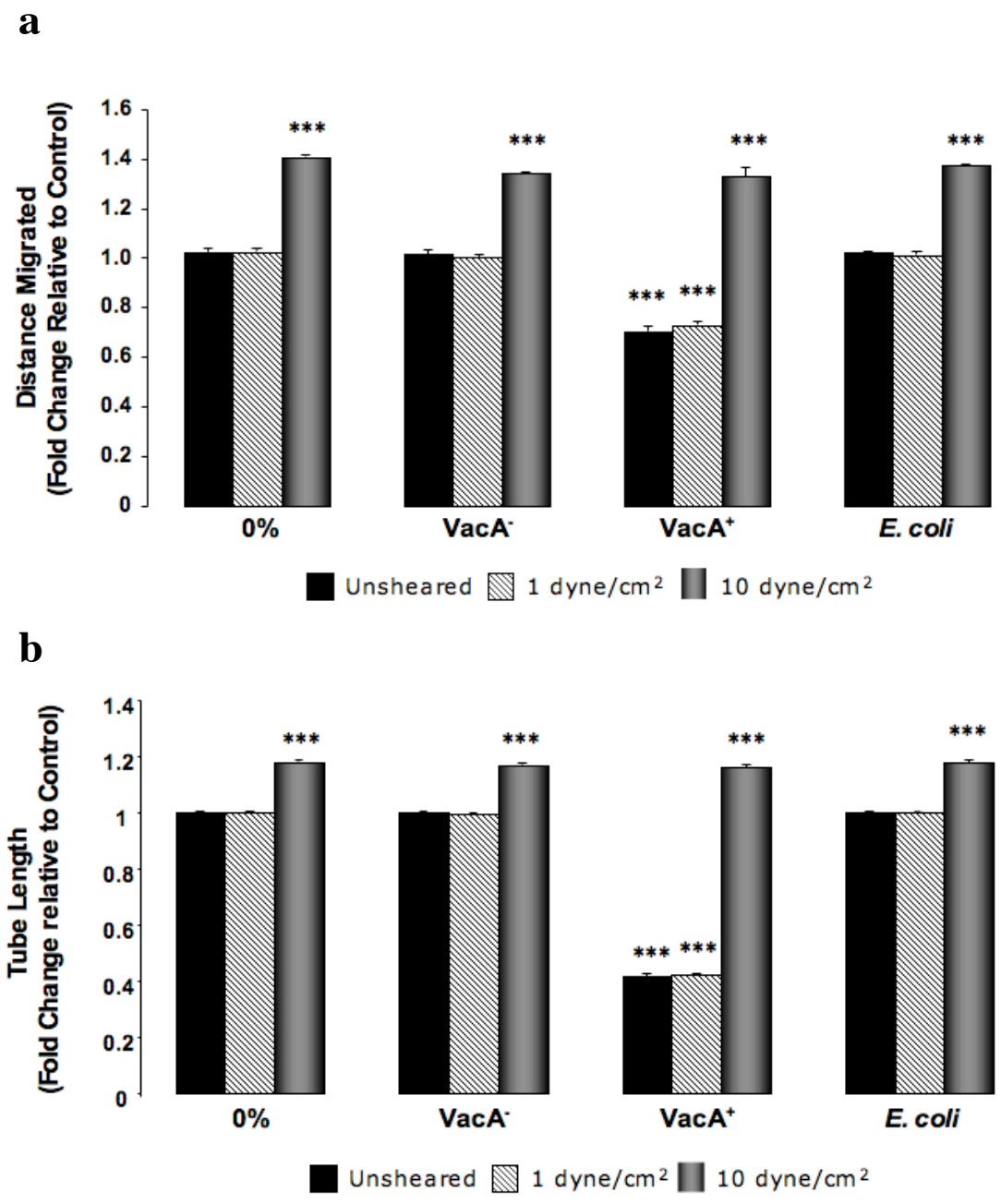


Fig. 5.2.2: Effect of *HPCM* and Shear on BAEC Migration and Tube Formation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) under shear conditions (1 and 10 dyne/cm^2) for 24 h, cells were monitored for (a) Migration and (b) Tube formation. *E. coli*- conditioned media was included as gram-negative bacterial control. Histograms represent fold change relative to unsheared 0% and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus unsheared 0%.

5.2.3 Laminar shear restores the VacA$^+$ *HPCM*-mediated decrease in BAEC proliferation- FACS Analysis.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$ strains) under shear (0- 10 dynes/cm^2) for 24 h, cells were monitored for proliferation by FACS analysis. VacA$^+$ *HPCM* reduced BAEC proliferation in comparison to control following 1 dyne/cm^2 shear for 24 h (Fig 5.2.3a). However, at 10 dyne/cm^2 no decrease in proliferation was observed (Fig. 5.2.3b). VacA$^-$ and *E. coli*- conditioned media had no effect on proliferation at either shear intensity.

5.2.4 Laminar shear restores the VacA$^+$ *HPCM*-mediated decrease in proliferation- cell counts.

Following BAEC exposure to 25% HPCM (VacA$^+$ and VacA$^-$ strains) under shear (0- 10 dynes/cm^2) for 24 h, cells were monitored for proliferation by cell counts. VacA$^+$ *HPCM* reduced BAEC proliferation to 0.65±0.01 and 0.63±0.006 fold of control following 0 and 1 dyne/cm^2 shear, respectively. At 10 dyne/cm^2, VacA$^+$ *HPCM* had no effect on migration relative to sheared control. VacA$^-$ and *E. coli*- conditioned media had no effect on proliferation, regardless of shear intensity (Fig. 5.2.4).

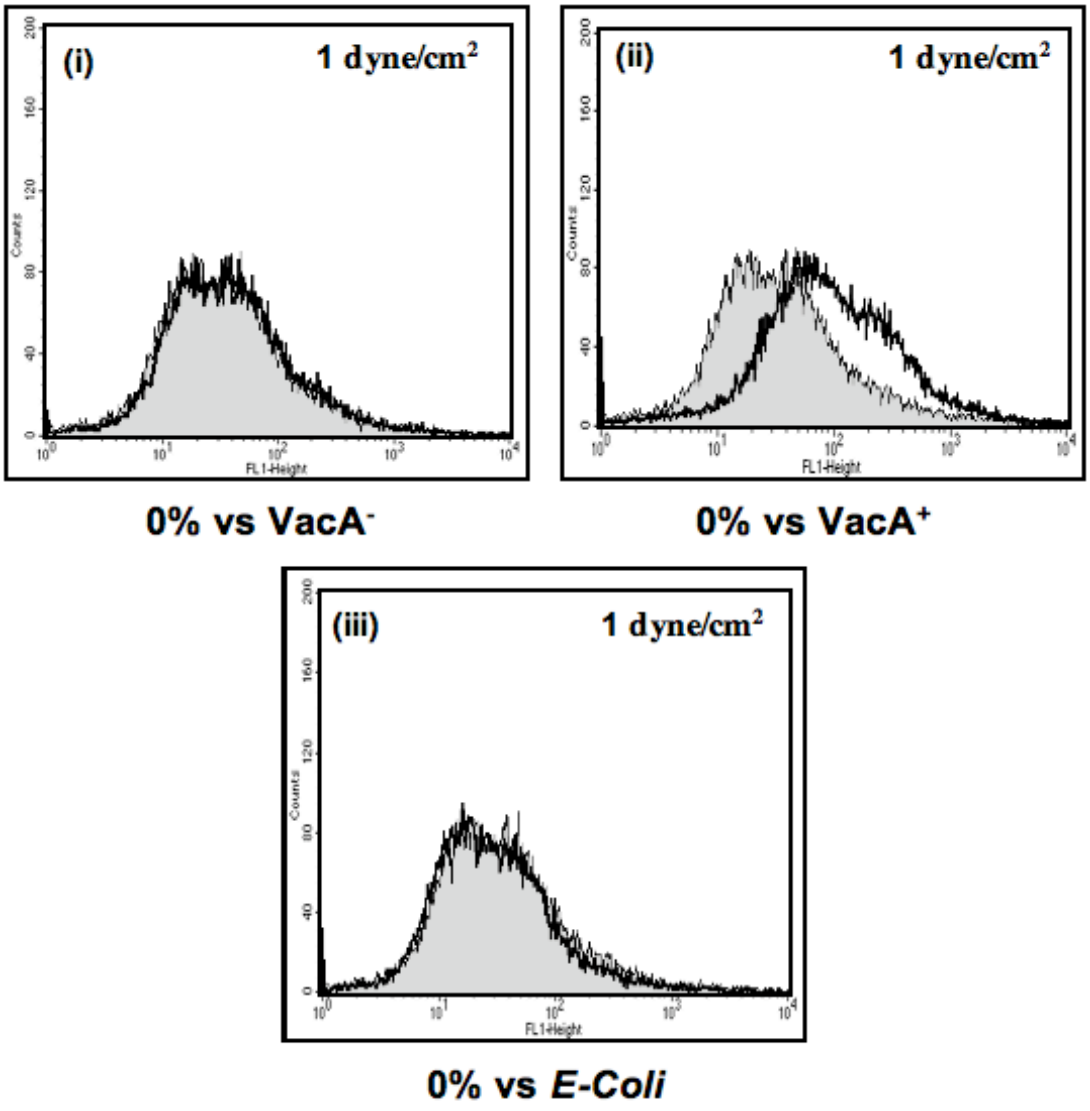


Fig. 5.2.3a: Effect of different *HPCM* strains and Low Shear on BAEC proliferation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and Vac^- strains) and 1 dyne/cm^2 shear for 24 h, cells were monitored for proliferation by FACS Analysis. Grey shaded area represents untreated control cells whilst black line represents conditioned media treated cells. (i) 0% vs. $VacA^-$ (ii) 0% vs. $VacA^+$ (iii) 0% vs. *E. coli*. Histograms are representative.

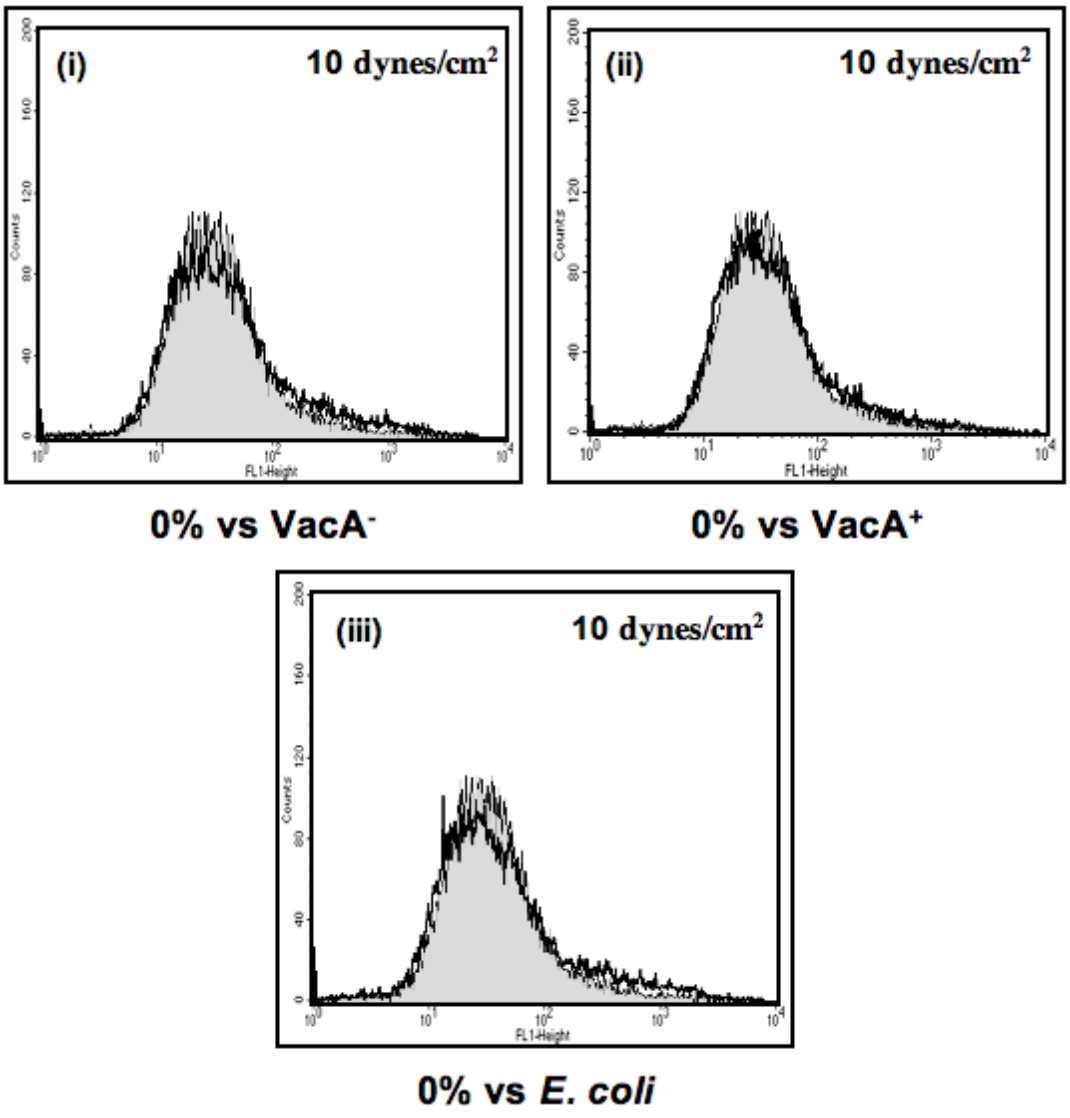


Fig. 5.2.3b: Effect of different *HPCM* strains and High Shear on BAEC proliferation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and Vac^- strains) and 10 dynes/cm^2 shear for 24 h, cells were monitored for proliferation by FACS Analysis. Grey shaded area represents untreated control cells whilst black line represents conditioned media treated cells. (i) 0% vs. $VacA^-$ (ii) 0% vs. $VacA^+$ (iii) 0% vs. *E. coli*. Histograms are representative.

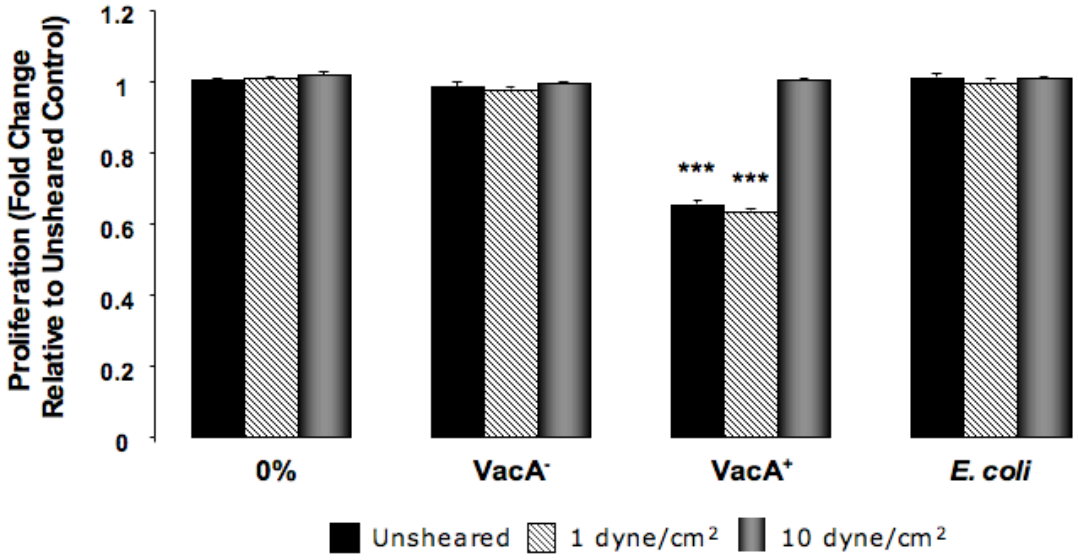


Fig. 5.2.4: Effect of *HPCM* and Shear on BAEC Proliferation. Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) under shear conditions (1 and 10 dyne/cm^2) for 24 h, cells were monitored for proliferation by cell counts. *E. coli*- conditioned media was included as gram-negative bacterial control. Histograms represent fold change relative to unsheared 0% and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus unsheared 0%.

5.2.5 Laminar shear restores the VacA$^+$ *HPCM*-mediated decrease in sodium nitrite production.

Following BAEC exposure to 25% *HPCM* (VacA$^+$ and VacA$^-$) under shear (0- 10 dynes/cm^2) for 24 h, cells were monitored for sodium nitrite production by DAN assay. Shearing at 10 dyne/cm^2 increased baseline levels of sodium nitrite. VacA$^+$ *HPCM* decreased sodium nitrite levels to 0.46±0.02 and 0.39±0.02 fold of 0% control at 0 and 1 dyne/cm^2, respectively, although shearing restored sodium nitrite levels to baseline control levels seen at 10 dyne/cm^2. VacA$^-$ *HPCM* and *E. coli*- conditioned media had no effects relative to 0% controls (Fig. 5.2.5).

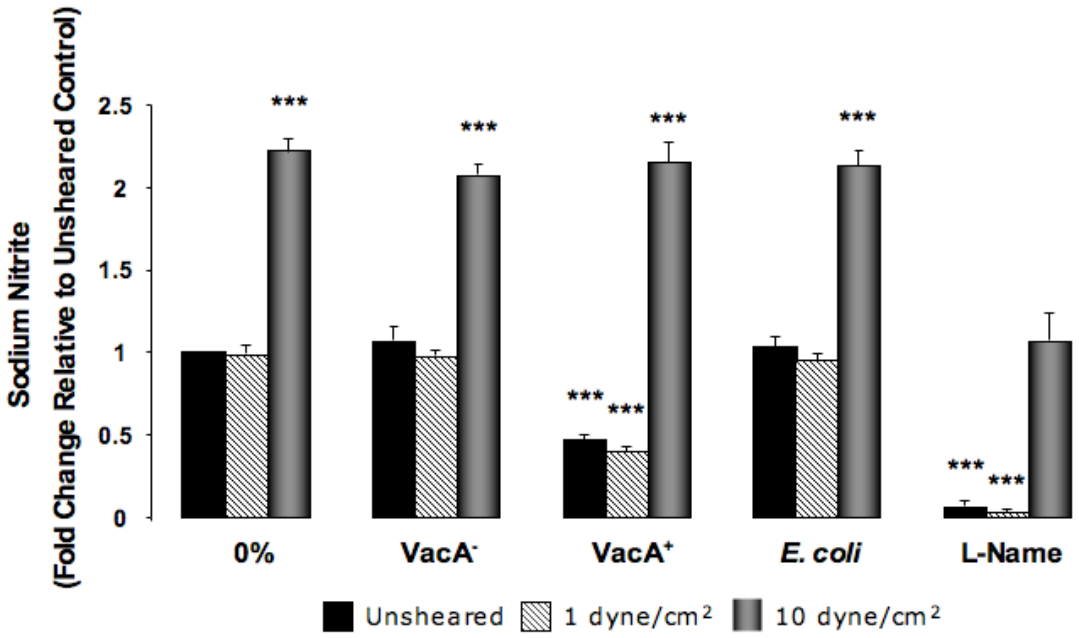


Fig. 5.2.5: Effect of *HPCM* on BAEC Nitrite production Following BAEC exposure to 25% *HPCM* ($VacA^+$ and $VacA^-$ strains) under shear conditions (1 and 10 dynes/cm^2) for 24 h, cells were monitored for sodium nitrite production by DAN assay. *E. coli*- conditioned media included as gram-negative bacterial control, L-Name included as negative control. Histograms represent fold change relative to unsheared 0% and are averaged from three independent experiments ±SEM. ***$P \leq 0.005$ versus unsheared 0%..

5.3 Discussion

The shearing effect exerted on the endothelial layer by blood flowing through the cardiovascular system results in the production of many anti-thrombotic and vasodilatory factors central to the maintenance of vessel homeostasis (van Thienen *et al.*, 2006). One such factor is NO, whose production is stimulated by laminar shear-dependent ion channel activation in endothelial cells (Olesen *et al.*, 1988). In addition, shear controls endothelial cell properties and function via transduction of signals to the cell through transmembrane structures classed as mechanotransducers (Chien, 2007) (i.e. integrins, ion channels, G-proteins and receptor tyrosine kinases). In this manner, healthy endothelial function is promoted.

In regions of the vasculature such as artery bifurcations or vessel curvature, normal laminar shear is perturbed and turbulent. As a result, NO production is lowered, control over endothelial function is lessened, and cellular alignment prevented, resulting in weakened cell-cell contacts (Gimbrone, 1999b). Moreover, an inflammatory phenotype dominates; meaning molecules present in the bloodstream can interact with the vessel wall in a manner that is not possible at other locations in the vasculature.

It has been suggested that the bacterium *H. pylori* plays a role in the initiation and development of an atherosclerotic plaque (Grabczewska *et al.*, 2006, Kowalski *et al.*, 2002), and may do so by exacerbating the pro-atherogenic effects associated with arterial bifurcations/vessel curvature. To date, no studies have looked at the effect of *H. pylori* under shearing conditions on cells of vascular origin. To address this, we have employed a robust and well characterised laminar shear model (Asada

et al., 2005, Dardik *et al*., 2005, Ley *et al*., 1989), to examine BAEC function in the presence of *HPCM*.

Our initial findings showed in accordance with Galbraith *et al*., that the shear stress-induced spatial reorganization of the actin cytoskeleton occurs after 24 h shear for realignment of endothelial cells (Galbraith *et al*., 1998). Moreover, cells exposed to low levels of shear and VacA$^+$ *HPCM,* showed marked decreases relative to control in migration and tube formation that were *not* present at high levels of shear. In addition, we saw a marked increase in both cell functions following 10 dyne/cm^2 shear for 24 h, as previously shown by Wu *et al.,* and Sprague *et al.,* for migration (Sprague *et al*., 1997, Wu *et al*., 1995) and our group for tube formation (Von Offenberg Sweeney *et al*., 2005). Similarly, the decrease in BAEC proliferation following VacA$^+$ *HPCM* treatment for 4 days at low shear was not present at high shear. Interestingly, no significant difference in proliferation was observed between low and high shear controls. This is in contrast to studies by Levesque *et al.,* who demonstrated a dose-dependent reduction of endothelial cell proliferation with increasing laminar shear stress (Levesque *et al*., 1990) after 48 h.

In the previous chapter, nitrite production- a marker of endothelial function, was examined. Here, a DAN assay was once again employed to study nitrite production in response to shear and it was found that the decrease in nitrite following incubation with VacA$^+$ *HPCM* at low shear was not present at high shear. In agreement with previous studies (Misko *et al*., 1993), a marked increase in nitrite levels following shear was observed.

These results suggest that at high levels of shear *HPCM* has little or no effect

on BAEC function, consistent with our knowledge that shear promotes an athero-protective phenotype. For example, normal laminar shear promotes the release of factors from endothelial cells that inhibit coagulation, migration of leukocytes and SMC proliferation, whilst also promoting cell survival (Traub and Berk, 1998). At low levels of shear such as those found at arterial bifurcations/curvature, incubation with VacA$^+$ *HPCM* causes endothelial dysfunction similar to that found in unsheared cells. Furthermore, the dysfunction coincides with a decrease in production of sodium nitrite, once again implicating *HPCM* with endothelial dysfunction in a nitrite-dependent manner.

However, the manner by which VacA reduces NO production has not yet been examined. It is possible that an indirect relationship exists between the two. For example, laminar flow opens K^+ and Cl^- ion channels, hyperpolarizing the cell and increasing the driving force for Ca^{2+} entry and NOS III activation. As previously discussed, VacA forms vacuoles by forming anion selective channels. Due to increased chloride conduction through these channels, intraluminal chloride concentrations are increased and vacuolar ATPase activity rises to compensate, resulting in an upsurge in proton pumping and a reduction in intraluminal pH. Here, it was shown that at low shear, NO production is lowered by VacA$^+$ *HPCM*, possibly due to the action of VacA increasing intraluminal chloride and vacolar ATPase concentrations, thus preventing the hyperpolarization of the cell necessary to activate the NOS III enzyme. Moreover, this may explain why VacA appears to have no effect at high shear rates- the increased opening of ion channels by shear results in a massive Ca^{2+} influx, quickly hyperpolarizing the cell, resulting in NO and

cGMP production- the latter of which inhibits further calcium entry into the cell. Here, the action of VacA may be prevented, as the large increase in chloride conduction due to opening of ion channels cannot be counterbalanced by the action of vacuolar ATPase, thus preventing the formation of vacuoles. It should be noted that this is speculative, but experiments examining whether VacA prevents hyperpolarization could provide some evidence for the theory.

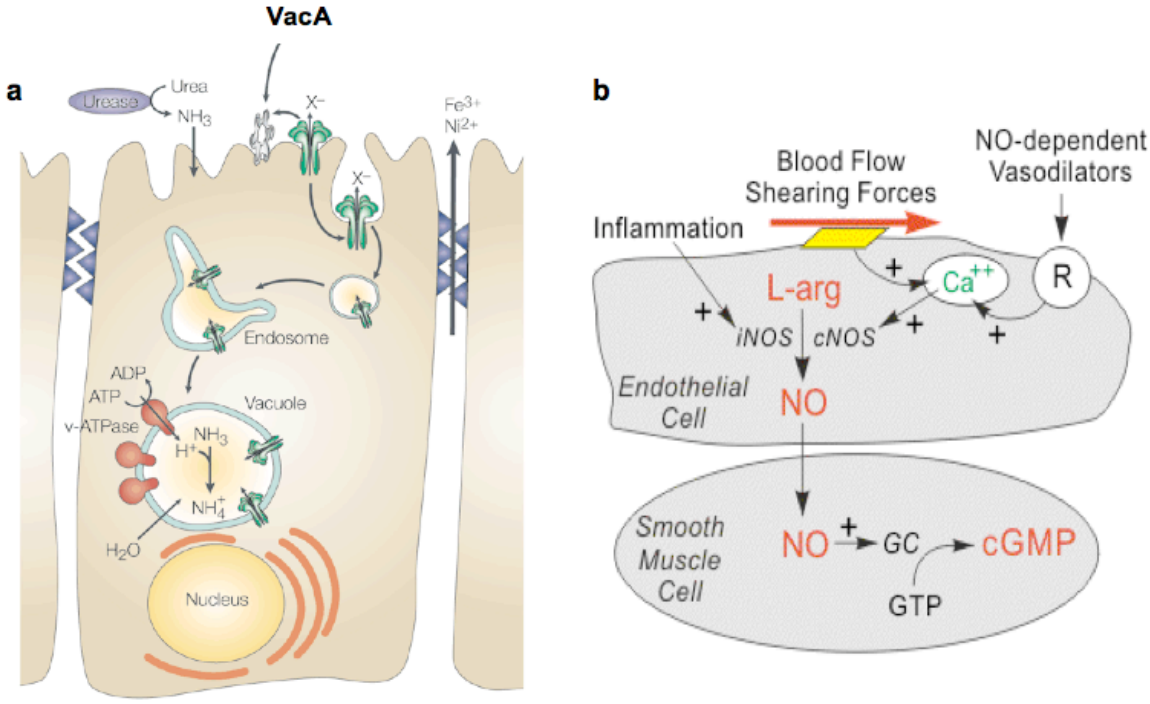


Fig. 5.3.1: The action of VacA and NO. (a) VacA binds to the cell and inserts into the plasma membrane, forming an anion-selective channel of low conductance. The toxin channels are slowly endocytosed and eventually reach late endosomal compartments, increasing their permeability to anions with enhancement of the electrogenic vacuolar ATPase (v-ATPase) proton pump. In the presence of weak bases, osmotically active acidotropic ions will accumulate in the endosomes. This leads to water influx and vesicle swelling, an essential step in vacuole formation (b) Laminar flow opens specialised ion channels within the endothelial cell membrane such as Ca^{2+}- activated K^{+} channels. This hyperpolarises the cell, increasing the driving force for Ca^{2+} entry and activating NOS III (eNOS) and hence NO production. Images (a) (Montecucco and Rappuoli, 2001) (b) (Klabunde R. E., 2007)

In conclusion, it was shown that high levels of shear can prevent the VacA *HPCM*-mediated dysfunction in endothelial cells *in vitro*. Low levels of shear, such as those found at arterial branch points/curvatures, were not protective. This data highlights the possible role of *H. pylori* in endothelial cell dysfunction at arterial branch points and curvatures.

Chapter 6

Final Summary

6.1 Final Summary

Cardiovascular disease is the number one cause of death globally, accounting for approximately 16.7 million deaths annually (World Health Organisation). It refers to any abnormal condition characterized by the dysfunction of the heart or blood vessels, and includes coronary heart disease, cerebrovascular disease, hypertension, rheumatic and congenital heart disease and heart failure. If the current trends in health continue, it is predicted that by 2015, 20 million people will die each year from CVD. To this end, improving treatments and identifying risk factors of the disease have become crucial, and are the focus of much study.

From such work, the importance of the endothelium has been identified in the initiation and progression of CVD (Pechanova and Simko, 2007), and in particular, the paradigm for our study, endothelial dysfunction leading to atherosclerosis. The endothelial cells lining the innermost layer of the vasculature are constantly exposed not only to any foreign matter in the bloodstream, but also to the strain and shear of blood flow. The laminar shearing effect of blood flow is responsible for stimulating the production of numerous endothelial factors vital to the maintenance of vessel homeostasis, such as those necessary for vasodilation and vasoconstriction (Gori *et al*., 2007). In addition, normal endothelial functions include mediation of coagulation, platelet adhesion, immune function, and control of volume and electrolyte content of the intra- and extra-vascular spaces (Strukova, 2006). Importantly, shear also regulates endothelial cell functions through

mechanotransducers such as integrins, ion channels, G-proteins and receptor tyrosine kinases (White and Frangos, 2007).

Loss of control over these processes, resulting in endothelial dysfunction, is considered pivotal to the initiation of atherosclerosis, and has been shown to occur at arterial bifurcations/curvatures (Cunningham and Gotlieb, 2005). Here, perturbed shear predominates, reducing cellular production of homeostatic factors such as nitric oxide, resulting in weakened cell-cell contacts, lessened cell functional control, and upregulation of pro-inflammatory molecules. When these events occur in the presence of CVD risk factors, initiation of an atherogenic plaque may ensue (Malek *et al*., 1999).

There are many traditional risk factors for CVD, including high cholesterol and blood pressure, obesity and smoking. Moreover, research has also identified many non-traditional factors such as elevated C-reactive protein levels (Schwedler *et al*., 2007), homocysteine levels (Caramia and Belardinelli, 2006), and bacterial infection. In particluar, the bacterium *H. pylori* has been identified in such a role, with many groups supporting the association (Hoffmeister *et al*., 2001, Majka *et al*., 2002, Pieniazek *et al*., 1999, Zito *et al*., 1999). To our knowledge however, no group has examined the effect of the bacterium or its secretions on cells of aortic or coronary origin, where a vascular occlusive pathology typically manifest. In order to address this our work has focused on the effects of *H. pylori* OMP and conditioned media on BAEC in the presence and absence of physiologically relevant levels of shear.

One of the major obstacles facing scientists examining a role for *H. pylori* in atherosclerosis is establishing a way that the bacteria can enter the blood stream and interact with the vessel wall. A 2003 paper by Semino Mora *et al.,* examining the location of *H. pylori* in the stomach provided insight on this. It was shown that the bacteria can leach from the lumen of the stomach into epithelial cells and the lamina propria (Semino-Mora *et al*., 2003), the latter of which contains gastric mucosal blood capillaries (Fig. 6.1.1). Here the bacterium could pass via diapedesis through the capillary wall and into the main circulatory system, with the ability of *H. pylori* to weaken epithelial cell-cell contacts supporting this action (Amieva *et al*., 2003, Krueger *et al*., 2007, Papini *et al*., 1998). In addition, a 2006 study by the same group reported that *H. pylori* can be found on the erythrocytes in capillaries and post-capillary venules in the gastric mucosa of humans and Rhesus monkeys. This association was mediated by binding of the SabA OMP adhesion to erythrocytes (Aspholm *et al*., 2006). The concept of bacterial leaching into the circulatory system is not alien. Periodontal infection of a chronic nature has frequently been shown to leach into the bloodstream and has even been implicated in atherosclerosis and CAD (Desvarieux *et al*., 2005, Epstein, 2002). Further to the theory of leaching, a number of studies have confirmed cross-reactivity between anti-*H. pylori* antibodies and blood vessel antigens. Franceschi *et al.,* reported that anti-CagA antibodies cross-reacted with antigens of both normal and atherosclerotic blood vessels by immunohistochemistry (Franceschi *et al*., 2002) while Guo *et al.,* showed anti-*H. pylori* antibodies cross-react with some antigens of human erythrocyte membrane (Guo *et al*., 2007). This data points to the presence of the bacterium in the

bloodstream, and thus a potential for it to be transported throughout the vasculature, possibly through an erythrocyte interaction.

As already mentioned, our premise encompasses the ability of *H. pylori* to enter the bloodstream and interact with the vessel wall, particularly at regions of perturbed/turbulent flow where the net shearing effect on the endothelium is lessened. It may be more likely however, that bacterial secretions from the stomach are involved in these events, as the immune system would rapidly detect and remove the *whole* bacterium from circulation. This hypothesis seems possible, as one such bacterial fraction- LPS, has been shown to elicit only a weak immune response by mimicking Lewis blood group antigens (Appelmelk *et al*., 1996). Moreover, the relative size of a secreted protein to the whole bacteria may mean it avoids detection in the bloodstream long enough to interact with the endothelium. For these reasons, we focused on OMP and secretory fractions of *H. pylori* in our studies.

Our experiments were carried out using the *H. pylori* 60190 human strain. It is associated with increased toxicity due to its CagA and VacA positivity, and is commonly used in many studies (Ganten *et al*., 2007, Ohno and Murano, 2007, Schwartz and Allen, 2006). Many different *H. pylori* strains have been used for experimental purposes, however cytotoxin-expressing strains correlate with increased pathogenicity and long-term gastric colonization. For example, Al-Marhoon *et al.,* report $CagA^+$ strains induces greater levels of prostaglandin E2 than $CagA^-$ (Al-Marhoon *et al*., 2004), Pillinger *et al.,* observed $CagA^+$ strain 147A stimulates gastric epithelial cell MMP-1 secretion when compared to $CagA^-$ Tx30a strain (Pillinger *et al*., 2007), and increased thrombin generation with $VacA^+$ strains

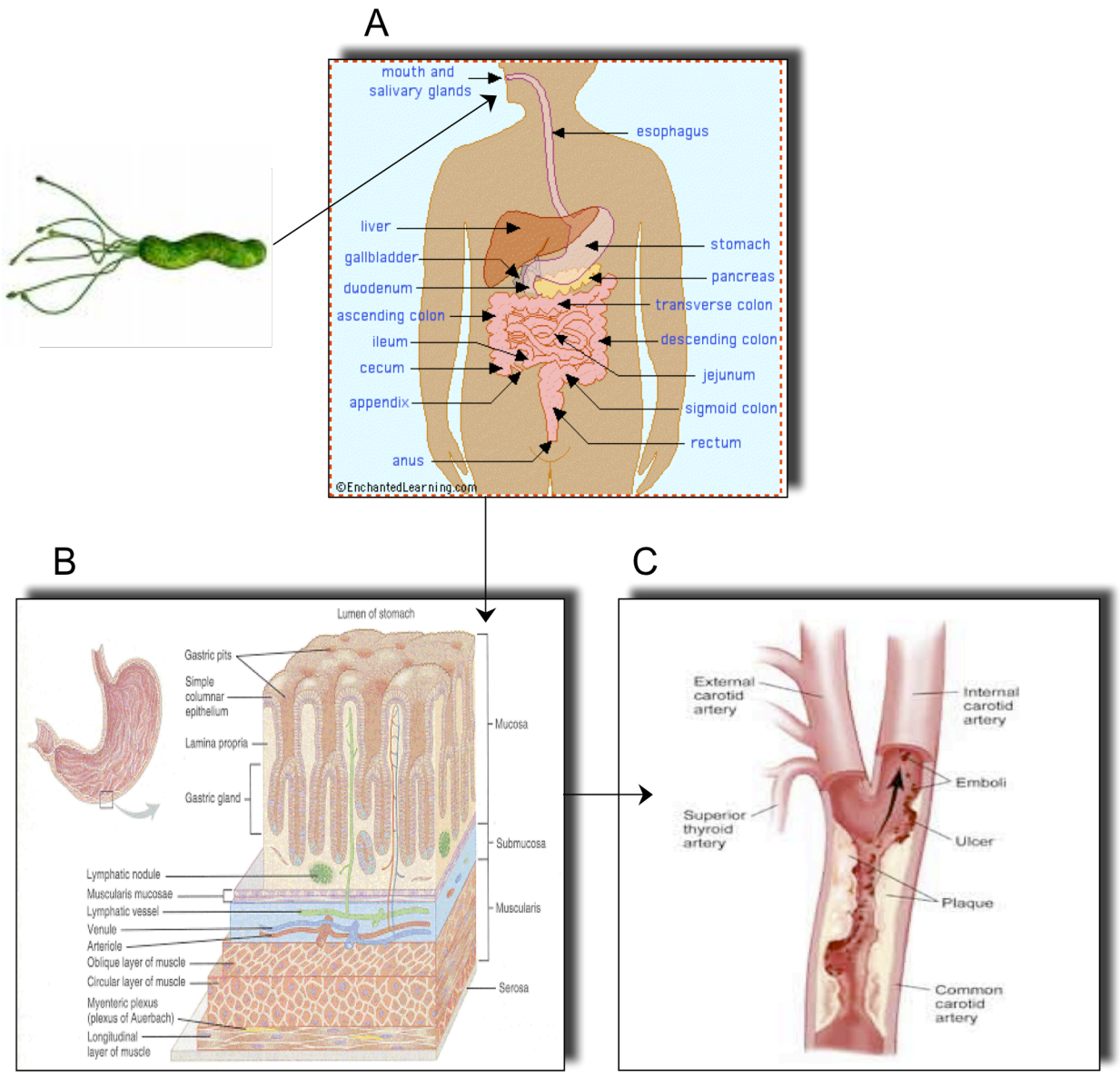


Fig. 6.1.1: *H. pylori,* from transmission to atherosclerosis. (a) *Helicobacter pylori* can be transmitted through person-to-person contact, contaminated drinking water, from animals such as cats or pigs, through the use of endoscopy techniques to examine gastric disorders. Following ingestion the bacterium progresses to the stomach; (b) The bacterium, leaches from the lumen of the stomach into epithelial cells and the lamina propria, where it may enter the capillaries; (c) *H. pylori* travels in the blood stream to arterial bifurcations, where it may interact with the endothelium. (Images (a) (EnchantedLearning, 2006) (b) (Tortora, 1996) (c) (Nott, 2006))

was shown by Suzuki *et al.* (Suzuki *et al*., 2005). Thus, a host of strains have been employed in previous studies; however, we chose the 60190 strain due to the large quantity of data available on its effects and also due to the availability of its VacA$^-$ mutant 60190:v1.

Our initial work focused on an OMP fraction isolated from *H. pylori* strain 60190, which we subsequently found to have little effect on endothelial function. An increase in BAEC tube formation and decrease in migration was observed, with a number of hypothesizes possibly explaining these opposing effects (Section 3.3). Our next series of experiments centered on bacterially conditioned media, with marked changes in cell function observed. Moreover, a role for VacA in this dysfunction was elucidated with data suggesting a VacA-dependent NO-reduction. Our final chapter explored the premise that *H. pylori* and in particular VacA, acts in regions of turbulent/perturbed shear to alter cell function. This would appear to be the case, with high shear apparently protecting against the NO-reducing effects of *HPCM*, albeit via an unknown mechanism. In order to further explore the latter, it is first necessary to understand the pathways through which NO impacts on cell properties and functions.

NO influences the cell-death programme of endothelial cells through mediation of *S*-nitrosylation of caspase-3, -8 and -9, resulting in the inactivation of each caspase (Kim *et al*., 2000, Torok *et al*., 2002) and hence, an anti-apoptotic effect (Hida *et al*., 2004). Importantly, *H. pylori* VacA induces apoptosis in gastric cells through activation of caspase-8 and 9 (Manente *et al*., 2007). Endothelial migration is increased following exposure to NO, via NO-mediated activation of the

PI3K/Akt pathways (Kawasaki *et al*., 2003). While no study has examined the effect of VacA specifically on the migratory pathway, PI3K activation by *H. pylori* has been shown on macrophage (Allen *et al*., 2005), and Il-6 induction by *H. pylori* requires TLR4-dependent NF-kappaB activation and mitogen- and stress-activated PI3K-triggered phosphorylation events (Pathak *et al*., 2006). This data suggests *H. pylori* could influence PI3K activation/inactivation in endothelial cells, possibly influencing the cells migratory phenotype. Both proliferative and angiogenic endothelial cell phenotypes are known to be influenced positively by NO, through VEGF/NO mediated activation of the MAPK pathway (Parenti *et al*., 1998, Zheng *et al*., 2006), although Ziche *et al.,* suggests basic fibroblast growth factor (bFGF) in concert with NO may also be involved (Ziche *et al*., 1997). Interestingly, VacA has been shown to activate the MAPK pathway in epithelial cells (Nakayama *et al*., 2004), and in contrast to our findings, stimulate proliferation of gastric cancer cells in this manner (Chen *et al*., 2006). Moreover, inhibition of T-cell activation and an increase in prostaglandin production through induction of cyclooxygenase-2 in gastric cells have also been observed in a VacA/MAPK dependent manner (Boncristiano *et al*., 2003, Hisatsune *et al*., 2007). Whilst these studies do not correlate with our own, all were carried out in gastric cells, not in aortic endothelial cells, perhaps accounting for the opposing effects on proliferation. Finally, eNOS has been shown to play a role in a VEGF-induced acute vascular permeability increase (Fukumura *et al*., 2001), possibly in a MAPK dependent manner. Notably, Caputo *et al.,* have demonstrated a VacA dependent up-regulation of VEGF expression in gastric cells (Caputo *et al*., 2003). Taken together these studies point to

a strong role for *H. pylori* in stimulating a VEGF/NO up-regulation in gastric cells. However, evidence from Liuba *et al.,* and now our group, suggests that NO production in endothelial cells, coronary and aortic respectively, is reduced by *H. pylori* (Liuba *et al*., 2003). As VEGF is often implicated in NO production and vice versa (Zheng *et al*., 2006), it seems likely that VEGF may also be down-regulated by *H. pylori* in endothelial cells. This would further contribute to the decreases we have observed in BAEC migration, tube formation, proliferation and nitrite production. An experiment examining VEGF expression in BAEC's following *HPCM* treatment may yield a definitive answer as to whether an indirect relationship between VacA and NO exists through VEGF.

While the association between *H. pylori* and NO appears promising, it would be remiss not to discuss other factors, which in association with VacA could induce the responses we have observed. Of particular note are the class of single membrane-spanning, non-catalytic Toll-like receptors (TLRs). TLRs are a form of pattern recognition receptor (PRR) that recognize structurally conserved molecules that are broadly shared by pathogens and are termed pathogen-associated molecular patterns (PAMPs). Following activation of TLRs by PAMPs, an immune response is triggered resulting in production of inflammatory cytokines. Numerous groups have examined whether *H. pylori* induces TLR activation, resulting in downstream cytokine expression. Uno *et al.,* observed in gastric cells that *H. pylori* LPS results in TLR2 activation through TLR4 signaling propagated through extracellular signal-regulated kinase (ERK) and NFkappaB activation (Uno *et al*., 2007). In addition, iNOS expression and resulting NO production was increased in cells following LPS

exposure. Interleukin-8 induction via TLR2 and MAPK pathway following exposure to *H. pylori* heat-shock protein 60 was shown by Zhao *et al.,* in human monocytes (Zhao *et al*., 2007). Most notably, Smith *et al.* observed a *H. pylori* and TLR2 induction of syndecan-4 expression in an NFkappaB-dependent manner. Syndecans are a family of trans-membrane heparin sulfate proteoglycans (HSPG) that have are known to affect a variety of biological functions, including the regulation of growth factor signaling, adhesion, tumourigenesis, and inflammation. These results indicated that the interaction between *H. pylori* and TLRs has many downstream effects, including iNOS induction and syndecan expression, which may account for the effects we have seen in BAECs. However, this seems unlikely as no experiments have been carried out examining the bacterium's effect on cellular functions and moreover, no studies have been conducted in endothelial cells of a coronary or aortic nature. What does appear plausible based on the aforementioned studies is a TLR-mediated interaction between *H. pylori* and NO, similar to the role we have proposed for VEGF. To confirm/disprove this theory, a series of experiments examining firstly, TLR activation in endothelial cells in response to *H. pylori* and secondly, whether blocking TLR signal transduction has any effect on the previously shown decrease in NO expression, following incubation with *H. pylori*.

Many aspects of *H. pylori's* interaction with the endothelium, and more so its putative role in atherosclerosis, remain unresolved. However, a number of studies could be undertaken to clarify these issues. In our own work, it was initially examined whether OMPs had any effect on BAEC function, and opposing changes in cell migration and tube formation were found. As previously mentioned,

experiments examining the activation profile of the integrins involved in migration over time and isolation/testing the functions of individual OMP's in an *in vivo* model should clarify this issue. Furthermore, as this is an total OMP fraction, isolating specific OMPs with known effects- such as SabA, OipA, HopA or LPS and testing their effects on cell migration/tube formation could pinpoint which (if any) is responsible for the observed endothelial dysfunction. Our second set of studies sought to determine whether *H. pylori* conditioned media altered BAEC function. The decrease in BAEC proliferation, as measured by cell counts and FACS analysis, proved particularly robust, and identification of what stage of the cell cycle *HPCM* arrests/slows growth would extend our knowledge on this subject. To this end, FACS analysis can be employed to examine markers specific to each phase of proliferation. In addition, mechanistic data from the pathways through which *HPCM* exerts its effects on cell function would contribute significantly to our research. For example, Real-time PCR or Western blotting could be used to examine altered expression/activation of MAPK following exposure to *HPCM*. This would provide information into *how* decreased proliferation/angiogenesis may be occurring, and lead on to experiments determining how binding of VacA with its receptors on BAECs results in dysregulation of the pathway. In our final chapter, it was investigated how shear, NO and *H. pylori* can influence endothelial function. While our shear model has been used and validated previously (Colgan et al., 2007b, Pearce et al., 1996), conducting our experiments in a perfused capillary system would introduce another dimension to our work with pulsatile shear, whilst also confirming our laminar studies. The perfused transcapillary culture apparatus

(Cellmax® Artificial Capillary System) consists of an enclosed bundle of 50 semi-permeable Pronectin™-coated polypropylene capillaries through which medium from a reservoir is pumped at a chosen flow rate. By electronically altering the flow rate, varying pulsatile flow rates and hence pulse heights (pressure) can be achieved in this system. A schematic is shown in Figure 6.1.2.

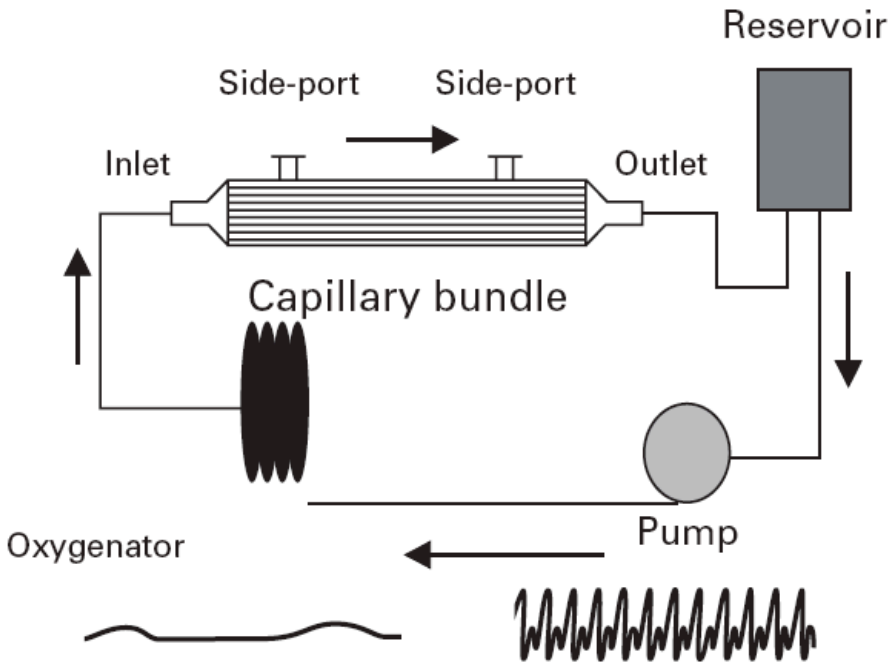


Figure 6.1.2 Schematic of the Cellmax® Artificial Capillary System: As the gear pump rotates, the motor shaft forces the pump pins to depress the pump tubing on the capillary module, thereby forcing culture media to flow in a pulsatile fashion through the gas-permeable silicone flow path tubing and through the capillary. By altering the flow rate using an electronic control unit housed outside the humidified incubator, varying pulsatile flow rates and hence pulse heights (pressure) can be achieved in this system. (Spectrum Laboratories, Calif., USA)

Endothelial dysfunction in conjunction with other cardiovascular risk factors, are hallmarks of atherosclerotic plaque formation. Another early indicator of a pro-atherogenic endothelium is inflammation. To extend our hypothesis on the role of *H. pylori* (and in particular VacA) in atherosclerosis the presence of inflammatory markers could be assessed following treatment. These may include C-reactive (Libby, 2002) and adhesion molecules such as ICAM, VCAM, P-selectin and E-selectin (all of which can be monitored by western blotting and/or FACS analysis).

In addition to bacterial fractions, another natural progression of our work could involve co-culturing of whole *H. pylori* with endothelial cells. In this manner, many aspects of the bacteria could be focused on that cannot be examined by bacterial fractions. A good example is CagA; it is not secreted into the extracellular matrix, but instead requires "docking" with host cell receptors with subsequent injection of the cytotoxin. Moreover, the ability of the bacterium to adhere to the endothelium under high or low shearing conditions could be elucidated. In this regard, low shearing rates, such as those found at arterial bifurcations/curvature could be predicted to enhance the ability of *H. pylori* to adhere to the endothelium.

While *H. pylori* has been implicated in cardiovascular disease, the bacteria *Chylamidia pneumonia*, has also been studied within this context. Both bacteria have been found in atherosclerotic plaques and are implicated in the initiation and progression of atherosclerosis (Witherell *et al*., 2003). However, as with *H. pylori* little or no data is available pertaining to its impact on endothelial function. A series of experiments such as those carried out by our group could easily be performed with conditioned media from both bacteria combined. Better still, a co-culture of

both bacteria with endothelial cells and subsequent examination cell function could increase our knowledge in this area.

Finally, all of these future studies are based on *in vitro* models. Ultimately, to prove these concepts an *in vivo* model must be employed. Perhaps the best place to begin is by elucidating and recording the manner in which *H. pylori* leaches through the gastric niche and enters the circulatory system. A 1995 study by Contag *et al.,* has provided the groundwork for such an experiment (Contag *et al*., 1995). In this study, three strains of Salmonella were bioluminescently labeled via transformation with a plasmid conferring constitutive expression of bacterial luciferase. Subsequently, detection of photons transmitted through tissues of animals infected with bioluminescent Salmonella allowed visualization of the bacteria localized to specific tissues through the IVIS50 system (Xenogen Corporation, Alameda, CA). A similar study could be employed to track *H. pylori* in real-time following feeding of infected chow to a mouse. Additionally, the experiment could be performed on ApoE knockout mice that are prone to developing atherosclerotic plaques to monitor whether the bacteria localizes to arterial bifurcations and interacts with the endothelium. This becomes particularly important in the light of papers for (Liuba *et al*., 2003) and against (Mach *et al*., 2002) the association of the bacterium and atherosclerosis in mice.

In conclusion, it was demonstrated that the VacA cytotoxin from *Helicobacter pylori* effects endothelial function in a pro-atherogenic manner, through reduced nitric oxide production. Moreover, it was shown that at high shear rates the endothelium was athero-protective and prevented these effects. Concomitantly, decreasing migratory and increasing tube formation phenotypes in endothelial cells following exposure to *H. pylori* OMP was observed. Whilst these experiments point to a distinct role for the

bacterium in atherosclerosis, much remains to be elucidated both *in vitro* and *in vivo*. However, our data represent a robust starting point. While the overall clinical relevance of such studies remains to be seen, undisputed identification of another risk factor for cardiovascular disease is paramount in improving its treatments and reaching the ultimate goal of a cure. It is the author's opinion that, as with many diseases, the onus is on each individual to avoid the risk factors of CVD, and maintain a healthy lifestyle. For factors that cannot be controlled by exercise or diet, such as our proposed risk (*H. pylori*), a vaccine against the disease could be administered at a young age, similar to the MMR vaccine. Research into such products is approaching fruition. Until then eradication therapies will continue to dominate and the bacteria will persist and thrive in the 10% of developed and 90% of developing countries.

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