Alkaline phosphatase

An old enzyme newly discovered

Implications in cardiac surgery

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Alkaline phosphatase

An old enzyme newly discovered

Implications in cardiac surgery

Alkalische phosphatase

Een oud enzym hernieuwd ontdekt

Implicaties voor de hartchirurgie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 25 november 2011 des ochtends te 10.30 uur

door

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geboren op 25 mei 1973
te Veendam

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Surgeons must be very careful when they take the knife!
Underneath their fine incisions stirs the culprit—LIFE!
(Emily Dickinson)

Voor Kees, Koen en Puck

Contents

Chapter 1

General introduction
Aim and outline of the thesis

The whole body inflammatory response to cardiopulmonary bypass

In 1953 J.H. Gibbon jr. was the first surgeon to use cardiopulmonary bypass (CPB) in cardiac surgery. He successfully repaired a large atrial septal defect in an 18-year-old woman [1]. Nowadays, fewer than 60 years after the first use of CPB hundreds of thousands of patients worldwide undergo successful CPB assisted cardiac surgery at low risk and with good clinical outcomes every year. In the Netherlands cardiac surgery is performed in sixteen hospitals where 12 to 15 thousand cardiac procedures are performed every year. The majority of cardiac surgery procedures are performed with the use of CPB.

Cardiopulmonary bypass is unique in that blood exposed to nonendothelial cell surfaces is collected and continuously recirculated throughout the entire body, subsequently resulting in a considerable activation of blood. In 1961 Lee described the denaturation of plasma proteins by CPB as a cause of morbidity and death [2]. Kirklin et al. [3] described the reaction of the body to CPB as a whole body inflammatory response, a collective term for a very complex reaction at cellular, humoral and metabolic level.

The whole body inflammatory response to CPB is both material dependent caused by exposure of blood to non physiologic surfaces and conditions and material independent caused by the surgical trauma itself, ischemia-reperfusion, changes in body temperature and the release of endotoxin. Thus, the whole body inflammatory response results from both endogenous insults ('danger' signals) and exogenous insults ('stranger' signals) [4;5]. Throughout the years lots of efforts have been undertaken to improve the biocompatibility of the currently used CPB machines, thereby diminishing the material dependent whole body inflammatory response. For example, van Oeveren et al. [6] reported on the deleterious effects of CPB and investigated the use of a membrane oxygenator as compared to a bubble oxygenator. They found less blood activation and as a consequence less blood loss and blood requirement when a membrane oxygenator was used. Other examples of improvement of the CPB circuit are the introduction of heparin-coated tubes in the CPB circuit [7], different arterial and cardiotomy filters and minimizing the CPB circuit, thereby minimizing blood-foreign surface contact and also minimizing the amount of priming solution needed [8].

In addition to the measures taken to diminish the material dependent whole body inflammatory response, lots of efforts have been undertaken to diminish the material independent whole body inflammatory response. In this regard, a lot of research has been done in pharmacological strategies to attenuate the detrimental effects of CPB. For example glucocorticoids have been studied widely, albeit with inconsistent results [9;10].

The role of endotoxin in the material independent whole body inflammatory response has been well investigated. The release of endotoxin first in the portal and later on in the systemic circulation initiates alternative complement pathway activation and has been shown to cause both direct myocardial dysfunction and pulmonary capillary damage [11]. The mechanism of endotoxin release in cardiac surgery is reported differently among different authors. A lot of work in this area was

done by Oudemans-van Straaten who defended her thesis on the role of gut-derived endotoxin in cardiac surgery in 1995 [12].
Endotoxin is produced by intestinal flora and is normally confined to the lumen of the intestine. During cardiopulmonary bypass (CPB) mesenteric hypoperfusion occurs which results in a loss of barrier function and as a consequence bacterial endotoxins may enter the systemic circulation [13-15]. The amount of endotoxin in the systemic circulation appears to be related to CPB time and also to cross clamp time[15]. An endotoxin molecule consists of four different parts (figure 1): a lipid A moiety, an inner core, an outer core and an O-antigen. The lipid A moiety of the endotoxin molecule is composed of two phosphorylated glucosamine saccharides. The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A [16].

In a similar way extracellular nucleotides like ATP and ADP leaking from cells and organs that are affected by ischemic injury have a profound inflammatory effect. Matzinger has described these potent inflammatory stimulators as 'danger' signals. In contrast, the above mentioned release of endotoxin might be addressed as 'stranger' signals [17]. Both stimulators of the inflammatory response act synergistic.

Alkaline phosphatase

Alkaline phosphatase is a ubiquitous endogenous ecto-enzyme in the human body. This ectophosphatase is widely expressed in many organs that are exposed directly or indirectly to the external environment, like the gastrointestinal tract and the lungs. A physiological role for alkaline phosphatase was proposed in 1997 by Poelstra et al [18]. Alkaline phosphatase dephosphorylates and thereby detoxifies endotoxins (lipopolysaccharides) but also extracellular nucleotides [19;20]. The phosphorylated lipid A moiety of endotoxin is a substrate for alkaline phosphatase, which enzymatically dephosphorylates the toxic lipid A part into monophosphoryl lipid A, which is non-toxic (Figure 1).

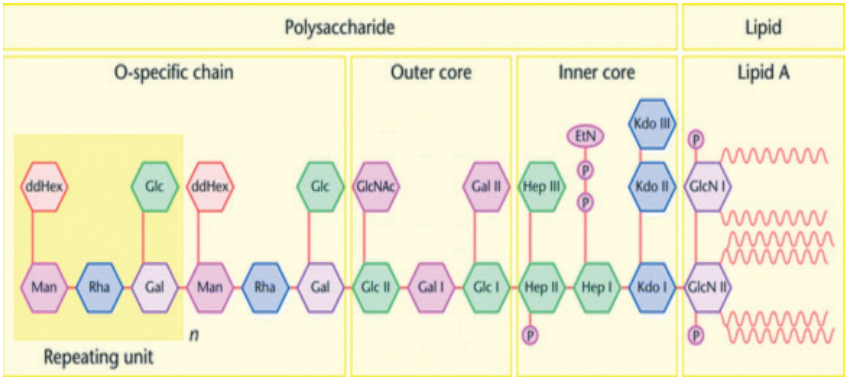

Figure 1 Chemical structure of endotoxin

Furthermore, alkaline phosphatase has been reported to dephosphorylate adenosine triphosphate (ATP) (Figure 2) into adenosine and thus converts nucleotides into non-inflammatory nucleosines [21;22]. The increase in adenosine signaling plays an important role in counterbalancing the deleterious effect of acute inflammation, including the attenuation of vascular leakage. Both endotoxins and nucleotides are potent inflammatory triggers and are sensed as 'stranger' or 'danger' signals to the innate immune system, and subsequent local and systemic inflammatory responses (SIRS) may result from the exposure to these pro-inflammatory signals [5].

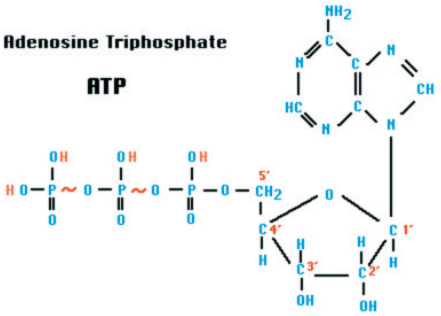

Figure 2 Chemical structure of ATP

In various animal studies promising therapeutic effects in reducing the inflammatory response with the use of intravenous alkaline phosphatase are shown [23-25]. In a clinical study in severe sepsis patients it was documented that continuous infusion of calf-intestinal alkaline phosphatase significantly improves renal function [26]. In patients with severe ulcerative colitis exogenous alkaline phosphatase improves short-term disease activity scores [27]. More recently, a protective role of intestinal alkaline phosphatase has been demonstrated in necrotizing enterocolitis in an experimental rat model [28].
In Figure 3 the proposed mechanism of action of alkaline phosphatase is demonstrated.

Aim and outline of the thesis

Based on the promising results of alkaline phosphatase in the attenuation of the inflammatory response in several studies we focused in this thesis on the effect of bovine Intestinal Alkaline Phosphatase (bIAP) on the inflammatory response in the field of cardiology and cardiac surgery. The study drug bIAP was manufactured by BBI/Biozyme ltd (Bleanavon, Wales, UK) and Alloksys Life Sciences B.V. (Bunnik, The Netherlands).
In **chapter 2** the pathophysiology and possible therapeutic strategies to attenuate the whole body inflammatory response after coronary artery bypass grafting (CABG) with the use of CPB are reviewed, focused on endotoxin release.

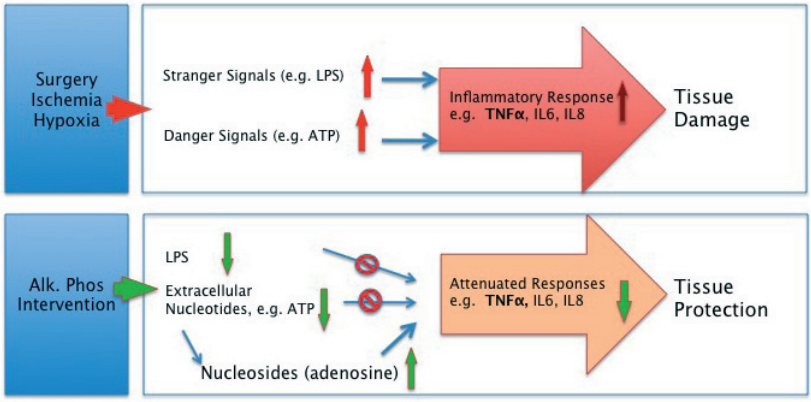

Figure 3 Proposed mechanism of action of alkaline phosphatase

Both material dependent and material independent factors in preventing the whole body inflammatory response are discussed.
To investigate whether bIAP has an anti-inflammatory effect in acute myocardial infarction, we used the left anterior descending (LAD) ligation model to induce acute myocardial infarction in mice. BIAP was given as a prophylaxis prior to LAD ligation and the inflammatory response was measured. The design and results of this study are presented in **chapter 3**. To investigate the potential effect of prophylactic bIAP treatment in patients undergoing elective Coronary Artery Bypass Grafting (CABG) with the use of CPB we performed a prospective, randomized, placebo controlled study (the APPIRED study, Alkaline Phosphatase in Prevention of Ischemia Reperfusion Damage) in which we administered bIAP intravenously as a bolus followed by continuous infusion for 36 hrs. Both inflammatory parameters and clinical outcome were assessed. The APPIRED study is described in **chapter 4**. In **chapter 5** a substudy of the APPIRED study is described. We focused on the level of alkaline phosphatase and the induction of endogenous alkaline phosphatase. Alkaline phosphatase levels are measured and endogenous alkaline phosphatase release is described. The mechanism of endogenous alkaline phosphatase release and its implications are discussed. We hypothesized that the release of endogenous alkaline phosphatase might be the result of the use of low dose aprotinin in the studied population. That is the reason why we performed a small pilot study in patients undergoing elective CABG with the use of CPB but without the use of low dose aprotinin. The results of this study are described in **chapter 6**. In an animal study in piglets different ways of administration of bIAP, as well as different dosing regimens and the associated pharmacokinetics are described. The primary endpoint of this study was to induce endogenous alkaline phosphatase release in the same way as we found it in CABG patients. The results of these piglet studies are described in **chapter 7**. **Chapter 8** consists of the general discussion and future perspectives. Finally, **chapter 9** provides the Dutch summary.

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Chapter 2

Endotoxin release in cardiac surgery with cardiopulmonary bypass: pathophysiology and possible therapeutic strategies. An update.

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Summary

Cardiac surgery with cardiopulmonary bypass provokes a systemic inflammatory response syndrome caused by the surgical trauma itself, blood contact with the non-physiological surfaces of the extracorporeal circuit, endotoxemia and ischemia. The role of endotoxin in the inflammatory response syndrome has been well investigated. In this report we reviewed recent advances in the understanding of the pathophysiology of the endotoxin release during cardiopulmonary bypass and the possible therapeutic strategies aimed to reduce the endotoxin release or to counteract the inflammatory effects of endotoxin. Although many different strategies to detoxifie endotoxins were evaluated, none of them were able to show statistically significant differences in clinical outcome.

1 Introduction

Cardiac surgery with cardiopulmonary bypass (CPB) provokes a systemic inflammatory response syndrome caused by the surgical trauma itself, blood contact with the non-physiological surfaces of the extracorporeal circuit, endotoxemia and ischemia. Thus both material-dependent and material-indepent factors contribute to the inflammatory response. This inflammatory reaction may contribute to the development of postoperative complications, including myocardial dysfunction, respiratory failure, renal and neurological dysfunction, bleeding disorders, altered liver function, and multi organ failure [1]. Over the years, many different strategies, including new pharmacological agents, CPB circuits and components and surgical techniques have been employed to minimize the inflammatory response post CPB. The role of endotoxin in the inflammatory response syndrome has been well investigated. The release of endotoxin first in the portal and later on in the systemic circulation initiates alternative complement pathway activation and has been shown to cause both direct myocardial dysfunction and pulmonary capillary damage [2]. This report will review recent advances in the understanding of the pathophysiology of the endotoxin release during cardiopulmonary bypass and the possible therapeutic strategies aimed to reduce the endotoxin release, or its effects.

2 Endotoxin

2.1 Endotoxin structure and activity

Bacteria are surrounded by a cell wall that guarantees the shape and the integrity of the microbial body. In Gram-negative bacteria this envelop represents the outer membrane, in which lipopolysaccharides or endotoxins are the main constituents

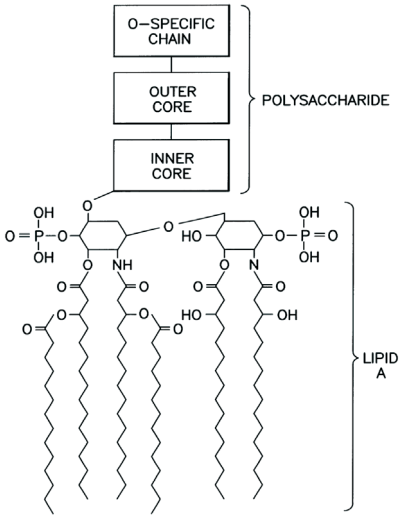

Figure 1 Schematic structure of endotoxin

and essential for bacterial growth and viability [3]. An endotoxin molecule consists of four different parts: a lipid A moiety, an inner core, an outer core and an O-antigen. The lipid A moiety of the endotoxin molecule is composed of two phosphorylated glucosamine saccharides. The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A (figure 1).
Lipid A containing only one phosphate group, monophosphoryl lipid A (MPLA) is non-toxic and able to attenuate the lethal effects of endotoxins[4].
Endotoxin is produced by intestinal flora and is normally confined to the lumen of the intestine by a barrier of endothelial cells. When entering the circulation, endotoxins bind to the lipopolysaccharide-binding protein (LBP) which interacts with various receptors, like Toll Like Receptor-4 (TLR-4) and leads to cytokine production [5] and thus to an inflammatory response.

2.2 Endotoxin and CPB

The release of endotoxins CPB has been studied widely in the 1990s. The mechanism of endotoxin release in cardiac surgery is reported differently among different authors. Andersen et al. [6] report that the endotoxins registered during CPB are mainly derived from environmental endotoxins. In 10 patients undergoing elective CABG, endotoxin samples were taken from the arterial outlet of the oxygenator, the pulmonary artery, the cardiac suction lines and the radial artery intraoperatively. Furthermore, fluid samples for endotoxin levels were taken from the cardioplegic fluid, the priming fluid, the blood transfusions and the ice used for external cooling of the heart. They reported the presence of endotoxins in the priming fluid of the CPB circuit and in the cardioplegic solution. Other authors also reported contamination with endotoxins by the extracorporeal set up, infusion solutions, drugs and surgical materials, such as instruments and gloves [7].
Rocke et al. [8] stated that during CPB hypoperfusion of the gut exists, which leads to increased intestinal permeability, allowing endotoxins to enter the portal circulation. In 1993 Ohri et al. [9] reported on increased gut permeability during CPB in 41 patients undergoing elective CABG, a valve operation or both. They measured alterations in gastric mucosal blood flow using a laser Doppler probe placed on the mucosa of the body of the stomach in 10 patients. Furthermore, they did small intestinal saccharide studies to assess active carrier-mediated, passive carrier-mediated, transcellular and paracellular transport respectively. They found a markedly imcreased lactulose/L-rhamnose gut permeability ratio after CPB ($p = 0.018$). A 48.7% reduction in gastric mucosal Doppler was found 30 minutes after the institution of CPB ($p = 0.0001$).
In 1994, the same group [10] reported on an animal study in 11 dogs where they did Doppler flow measurements, intramucosal pH by tonometry and oxygen utilization in a model of hypothermic CPB. In that study they confirmed there previous findings on decreased laser Doppler flow during CPB. They also demonstrated mucosal acidosis and villus tip ischemia as a sign of metabolic derangement. In 1996, Riddington et al. [11] studied intestinal hyperpermeability in 50 patients undergoing elective CABG or

valve replacement. Patients received chromium 51-labeled ethylenediaminetetraacetic acid (^{51}CR-EDTA) as a marker of intestinal permeability, a technique that had been validated in patients with inflammatory bowel disease [12]. They showed a large and varied increase in intestinal permeability to ^{51}CR-EDTA, which starts during CPB and is sustained for more than 24 hours postoperatively. In 1997, increased gut permeability during cardiac operations with the use of CPB was confirmed by Oudemans-van Straaten et al. [13;14]. To determine whether intestinal permeability increases during cardiac operations and whether the amount of endotoxin release is related to this increased hyperpermeability, they measured in 23 patients undergoing elective CABG the urinary excretion of L-rhamnose and cellobiose, which was administered orally just before surgery and on the fifth postoperative day. They measured an increased level of cellobiose in urine during CPB and a significantly related increased level of endotoxin in the blood as a result of increased intestinal permeability ($p < 0.01$). This increase in cellobiose was also significantly related to hypovolemia and the use of ephidrine during CPB. Ephidrine possibly leads to decreased splanchnic blood flow due to vasoconstriction.

Normally, systemic endotoxins are cleared from the circulation by Kupffer cells of the liver. During CPB however, Kupffer cell function can be suppressed by an overloading of the reticuloendothelial cells by cellular debris and aggregated proteins [15]. Moreover, during CPB a down-regulation of surface monocyte lipopolysaccharide-receptor CD-14, identified as the main endotoxin receptor on leucocytes occurs, which might lead to an increase in circulating endotoxin [16].

During cardiac surgery with the use of CPB pericardial pooled blood is returned to the CPB by cardiotomy suction catheters. In 18 patients undergoing elective CABG Spanier et al. [17] investigated levels of endotoxin in pericardial shed blood, which was pooled in the pericardial space for 45 minutes after placing of the aortic cross clamp and then returned to the cardiotomy reservoir. Blood samples were taken from the pericardial shed blood and the arterial line at the same time, and before and after reinfusion of the pericardial shed blood in the CPB circuit. They found a significantly higher amount of endotoxin in the pericardial shed blood as compared to the blood from the arterial line, both before and after reinfusion of the pericardial shed blood to the CPB circuit ($p < 0.05$).

Endotoxin levels can be measured by the Limulus amoebocyte lysate (LAL) test described by Baek [18]. Different modifications of this test have been used in the various studies described in this review. Partly due to non-standardized tests, different amounts of endotoxin are reported, which hampers comparison between study protocols. Jansen et al.[15] described three peaks in endotoxin levels during cardiac surgery. The endotoxin levels were higher after induction of anaesthesia, immediately after the start of CPB and after release of the cross clamp. Other authors also reported a peak level of endotoxin after release of the cross clamp, during reperfusion [19]. During reperfusion the flow through the splanchnic bed increases, leading to an extra wash-out of endotoxins.

Videm et al. [20] described differences in endotoxaemia among various cardiac operations. In 136 (CABG $n = 79$, valve $n = 19$, CABG + valve $n = 30$, CABG + carotid artery surgery $n = 8$) patients, endotoxemia levels were determined. The endotoxin concentrations in the isolated valve-replacement group were significantly lower than in any of the other three groups ($p < 0.05$). Using multivariate regression analysis they demonstrated a significant correlation between the number of grafts and the amount of endotoxin measured. Furthermore, they didn't find a difference in endotoxin concentrations between the patients, who developed complications and those who recovered uneventfully ($p = 0.62$). From these findings Videm et al. concluded that there might be a correlation between the amount of atherosclerosis and the endotoxemia level. In this study, CPB duration and aortic occlusion time were not significantly related to endotoxemia levels. In contrast with Videm et al., Rocke et al. demonstrated a strong correlation between the change in endotoxin levels and the duration of cross-clamping and also the CPB time [8]. In nine patients undergoing cardiac operations requiring a prolonged period of CPB (mitral valve replacement (MVR) combined with aortic valve replacement (AVR), MVR alone, MVR combined with tricuspid valve repair or redo-CABG) endotoxin levels were determined. They found no correlation between the change in endotoxin concentration and intra-operative mean perfusion pressure, mean bypass flow rate, calculated systemic resistance, nasopharyngeal and rectal temperatures and arterial blood gas status. However, a strong correlation between endotoxin concentrations and CPB time and also aortic cross-clamping time ($p < 0.005$) was found.
In all the above mentioned studies, probably due to small sample sizes, it has not been possible to find a correlation between endotoxin levels and postoperative morbidity.

3 Humoral factors in endotoxin detoxification

3.1 Anti- inflammatory cytokines

As a potent trigger of inflammatory and immunological reactions, endotoxin activates humoral- and cellular mediator-producing systems including cytokine generation. The appearance of cytokines follows a characteristic pattern beginning with tumor necrosis factor-alpha (TNFα) activity. The peak of TNFα activity is followed by the appearance of other pro-inflammatory cytokines like interleukin (IL)-6 and IL-8. Endotoxemia elicits systemic counter-regulatory mechanisms ranging from neuroendocrine responses to cellular and soluble antagonists of pro-inflammatory activity [21]. Thus, the presence of endotoxins is identified by numerous receptors of innate immunity. Of these receptors LBP, CD-14 and TLR-4 play a crucial role in the identification of endotoxins. LBP and CD-14 are recognizing and binding endotoxins, thus enhancing the activation of the immune system [22]. LBP is recognized as a typical acute-phase protein principally synthesized by hepatocytes, while CD-14 molecules are either produced *de novo* as acute-phase protein or are released into body fluids by shedding from cell surfaces. In 40 patients Kudlova et al. [22]

followed LBP and CD-14 levels in CABG surgery either with or without CPB. A significant increase of LBP concentration was found at the first postoperative day in both groups, reaching maximum levels at the third postoperative day. In the on-pump group the maximum CD-14 level was reached the first postoperative day, while, in the off-pump group the maximum CD-14 level was reached at the third postoperative day. Comparing CD-14 and LBP levels to known acute-phase proteins as C-reactive protein and long pentraxin showed no evidence for CD-14 and LBP serving as one of the acute phase proteins.

Grundmann et al. [23] investigated in 10 patients undergoing elective CABG the role of humoral factors for attenuation of the pro-inflammatory cytokine response to endotoxin stimulation during CPB. They found that during CPB a spontaneous production of anti-inflammatory cytokines such as IL-10 and IL-1 was induced, whereas only small amounts of the prototypical pro-inflammatory cytokines TNFα and IL-1β were measured. This anti-inflammatory plasma activity seemed to result partially from high circulating catecholamines and could contribute to the attenuation of the systemic inflammatory response to CPB.

Tolerance to endotoxin, with an important contribution of macrophages, has been defined in terms of reduced fever in experimental models after repeated injections of endotoxin. *In vitro* tolerance to endotoxin was described by Fitting et al. [24]. Tolerance to endotoxin was evidenced by a decreased capacity of different cells to respond to *in vitro* challenge with endotoxin. They found in mice injected intravenously with endotoxin, that tolerance to endotoxin is compartmentalized and that bronchoalveolar cells are less likely than, for example, splenocytes and peritoneal cells to develop tolerance to endotoxin, which might be an explanation for the fact that the lungs are a major place of inflammation during infection.

3.2 Anti-endotoxin antibodies (EndoCab)

From early foetal life, each individual has a certain amount of endogenous endotoxin immunity by maternal transfer during pregnancy. The amount of endotoxin immunity is enhanced by subsequent exposure to endotoxin [25]. Antibodies against the inner core region of endotoxins, respectively, immunoglobulin G (IgG) EndoCab and IgM EndoCab, can be measured by an enzyme-linked immunosorbent assay (ELISA) [26]. Bennett-Guerrero et al. were the first to perform a large descriptive trial in 301 patients undergoing CABG and/or valvular heart surgery, evaluating the association between preoperative anti-endotoxin immune status and morbidity following cardiac surgery [27]. Major complications were defined as in-hospital death or length of hospital stay greater than 10 days. Lower serum IgM EndoCab independently predicted an increased risk of major complications ($p = 0.002$). By contrast IgG EndoCab and total IgM levels did not predict outcome. Low preoperative IgM EndoCab may account for the significant proportion of the variability in outcome seen among patients with identical preoperative risk scores. Hamilton-Davies et al. [28] also demonstrated that low preoperative EndoCab levels

were related to poor outcome in valve-replacement surgery. In an observational study among 59 consecutive patients undergoing cardiac valve replacement they measured preoperative median IgG and IgM EndoCab levels as well as direct postoperative, and at 4 and 24 h postoperative levels. Of the 59 patients. 12 developed developed at least one complication. Of these 12, all had preoperative significantly lower IgM EndoCab levels as compared to the patients who recovered uneventfully ($p < 0.025$). Mathew et al. [29] demonstrated in 460 patients undergoing elective CABG, an association between low preoperative EndoCab levels and increased cognitive dysfunction postoperatively, especially in patients over 60 years old. Rothenburger et al. [30] investigated the relationship between IgG and IgM EndoCab levels and cytokine release and ventilation time in 100 patients undergoing elective CABG. They demonstrated significantly lower preoperative EndoCab levels in 15 patients who needed prolonged ventilation (p <0.001) with increased endotoxin and IL-8 levels direct postoperatively, and increased IL-6 levels 3 hours postoperatively ($p < 0.001$). Moreover, Moretti et al. [31] demonstrated in 474 patients undergoing CABG that lower preoperative serum IgM EndoCab level is a significant predictor of long-term mortality, independent of other risk factors (hazard ratio (HR) 0.73; 95% confidence interval (CI) 0.53-0.99; $p = 0.04$). Kaplan-Meier 5-year survival curves illustrated significantly lower survival for the group with low EndoCab levels. More recently Down et al. [32] showed in a retrospective study different levels of EndoCab in different populations and different countries, reflecting genetic and environmental variability between patient groups.

4 Pharmacological strategies to reduce endotoxin-mediated inflammation

4.1 Corticosteroids

The influence of prophylactic corticosteroid infusion during CPB, on the inflammatory response and on the postoperative course, is controversial. Some authors have demonstrated that treatment of patients with a large dose of corticosteroids attenuates the CPB-induced systemic inflammatory reaction in patients undergoing cardiac surgery. Thus, corticosteroids can reduce the complement-mediated activation of neutrophils and inhibit the secretion of pro-inflammatory cytokines, including TNF-α and IL-6 [33;34]. Bourbon et al. demonstrated that low dose methylprednisolone also reduces the inflammatory reaction after CPB, by inhibition of pro-inflammatory cytokines [35]. A total of 36 patients undergoing elective CABG were devided into three groups: a control group, a group receiving 5 mg kg^{-1} methylprednisolone, and a group receiving 10 mg kg^{-1} methylprednisolone just at the start of CPB. Plasma levels of IL-6 and TNFα were determined before, during, and after CPB as well as oxygen free radical (OFR) production. They found a significant increase in cytokine release and OFR after CPB. Cytokine release was significantly reduced with 5 mg kg^{-1} methylprednisolone ($p < 0.05$). Moreover, OFR release was significantly reduced with a greater dose of

methylprednisolone (10 mg kg^{-1}). However, Chaney et al. [36] investigated high-dose methylprednisolone in 30 patients undergoing elective CABG. They administered 30 mg kg^{-1} methylprednisolone during sternotomy and 30 mg kg^{-1} methylprednisolone during the initiation of CPB. They measured alveolar-arterial oxygen gradient, lung compliance, shunt, and dead space at four times perioperatively. Postoperative tracheal extubation was performed at the earliest appropriate time. They found that high-dose methylprednisolone during CPB was associated with a significant increase in post-operative alveolar-arterial oxygen gradient and shunt and a prolonged time of tracheal intubation as compared to 30 patients in the control group receiving no methylprednisolone ($p = 0.05$). Karlstad et al. [37] investigated the influence of methylprednisolone on the endotoxin release during CPB. They measured endotoxin release in 13 patients undergoing CABG at different time points and randomized between methylprednisolone infusion (1 g per patient) at induction and no methylprednisolone. They found a significant rise in plasma endotoxin levels following the initiation of CPB and at removal of the aortic cross-clamp ($p < 0.05$), but they showed no differences in endotoxin release in the methylprednisolone-treated group as compared to the control group ($p = 0.68$). However, Wan et al. demonstrated that, in patients undergoing CABG methylprednisolone might reduce the endotoxin release [38]. They randomized 20 patients undergoing elective CABG to receive either 30 mg kg^{-1} methylprednisolone or matching placebo. They measured endotoxin levels in the superior vena cava and in the inferior vena cava at different time points. They found higher levels of endotoxin in the inferior vena cava as compared with the superior vena cava and a reduced level of endotoxin in the inferior vena cava in the methylprednisolone-treated group ($p < 0.01$). In both studies the small number of patients was a study limiting factor.

4.2 Ketanserin

The role of ketanserin, an inhibitor of serotonin-induced vasoconstriction and a weak α_1 sympathetic blocker, in the reduction of endotoxemia during CPB was investigated by Oudemans-van Straaten et al. [39]. In 29 patients undergoing elective CABG, either 0.1 mg kg^{-1} h^{-1} ketanserin intravenously for 4 h, starting at the induction of anaesthesia, or matching placebo was administered. To limit the hypotensive effect of ketanserin the dose was reduced to 0.05 mg kg^{-1} h^{-1} after 4 h. Circulating endotoxin was lower in patients treated with ketanserin ($p > 0.05$). They found that ketanserin administration during cardiac surgery might reduce but not abolish the release of endotoxin. Ketanserin appeared to be more protective at the onset of CPB than at the end. The most plausible reason for this phenomenon might be vasoconstriction leading to improved splanchnic microcirculation.

4.3 Taurolidine

Taurolidine is a tauramide derivate. The active metabolite of taurolidine is taurine, which has been identified as a 'balancing factor' in the glutamate system to modulate

and stabilize calcium homeostasis [40]. Doddakula et al. [41] reported on taurolidine, which was intravenously administered at the time of aortic clamping and at 12 and 24 h after unclamping. A total of 60 patiens undergoing elective CABG were randomized in four different groups. Thirty patients received 250 ml 2% taurolidine intravenously, 15 with crystalloid cardioplegia, and 15 received blood cardioplegia. Thirty patients received matching placebo. Pro- and anti-inflammatory cytokines IL-6 and IL-10 were determined at several time points as well as the occurrence of arrhythmias postoperatively. Administration of taurolidine in crystalloid cardioplegia patients resulted in a significant decrease in serum IL-6 and an increase in serum IL-10 at 24 hours post-unclamping compared to placebo ($p < 0.0001$). In addition, in the bloodcardioplegia group, there was a trend in decrease of IL-6 ($p = 0.068$). Further, postoperative arrhythmias were significantly reduced in the crystalloid cardioplegia with taurolidine group as compared with the placebo group ($p < 0.003$). In the blood cardioplegia with taurolidine group, there was no significant decrease in arrhythmias, although there was a trend towards a decrease ($p = 0.583$). In this study clinical significance could not be demonstrated.

4.4 Selective digestive decontamination

Selective digestive decontamination (SDD) by pharyngeal and gastric application of nonabsorbable antibiotics aims to act against Gram-negative aerobic bacteria, while leaving indigenous anaerobic microflora intact. Although the role of SDD in standard intensive care unit (ICU) patients remains controversial, a recent study of de Smet et al. [42] showed that mortality rates in standard ICU patients were reduced by 3,5 percent when SDD was applied as compared with the mortality rate associated with standard care ($p = 0.02$). The underlying theory for the use of SDD in cardiac surgery lies in the diminishing of the number of Gram-negative microorganisms in the gut leading to less ischemia-associated translocation of these bacteria and thus leading to a reduction in endotoxin release. In 1993, Martinez-Pellus et al. [43] were the first to investigate the role of SDD in reduction of endotoxemia during CPB. In a multicentre study among 80 patients undergoing either CABG or valvular surgery, patients received oral nonabsorbable antibiotics every six h from admission until their surgery or did not receive this treatment. The antibiotics consisted of a mixture containing amphotericin B, polymyxin E and tobramycin in sterilized water. Assessment of decontamination was performed by rectal swabs along with the measurement of circulating endotoxins, TNFα and IL-6. They found significantly lower values of endotoxin in the SDD treated group ($p = 0.01$). Nonsignificant differences were found in TNFα levels. In fully decontaminated patients, IL-6 levels were significantly lower after reperfusion ($p < 0.05$). However, they found no differences in clinical outcome, defined as postoperative fever, length of stay and in-hospital mortality, between the two groups. In contrast with these findings Bouter et al. [44] found no reduction in perioperative endotoxemia and cytokine activation when SDD was used. A total of 44 patients undergoing cardiac surgery (CABG, valve,

CABG + valve or other) were randomized to preoperative administration for 5-7 days of oral nonabsorbable antibiotics (polymyxin B and neomycin) or placebo. They also did rectal swabs and measurement of endotoxin and cytokines TNFα, IL-10 and IL-6. SDD significantly reduced the number of rectal swabs that grew aerobic Gram-negative bacteria ($p < 0.001$). SDD did not affect the occurrence of perioperative endotoxemia, nor did it reduce TNFα, IL-10 or IL-6 levels ($p > 0.20$). They also did not find any difference in clinical outcome, defined as length of stay and in-hospital mortality, when SDD was used.
The use of a laxant to clear the bowel from bacterial load was investigated by Taggart et al. [45]. In this study, 60 patients were divided into four groups: preoperative laxative with pulsatile, or non pulsatile flow during CPB, or no laxative with pulsatile, or non pulsatile flow during CPB. The laxative Picolax consisted of cathartic sodium picosulfate and an osmotic laxative, magnesium citrate. They found a gradual increase in endotoxin concentration during CPB as compared with baseline levels ($p < 0.001$), independently of the laxative used or different flow modes.

4.5 Inhibition of TLR-4 signalling

The main toxic part of endotoxins is the lipid A part in which the two phosphate groups are essential for many biological activities (figure1). By contrast MPLA is not toxic and even capable of inducing tolerance to subsequent endotoxin exposure. An IL-1-like receptor called TLR-4 is the transmembrane protein receptor for endotoxins that mediates cellular activation.

4.5.1 Eritoran, a lipid A antagonist

Eritoran was intended to be an endotoxin antagonist and it has been shown to inhibit TLR-4-mediated cell stimulation and to be an effective inhibitor of the toxic effects of endotoxins in animal models [46]. Bennett-Guerrero et al. [47] investigated in a double blind, placebo controlled study in 152 patients undergoing elective CABG with or without valve surgery with an ascending dose Eritoran, its effect on endotoxin-induced systemic inflammation. They found no statistically significant differences in most variables related to systemic inflammation or organic dysfunction. Hence, they concluded that blocking lipid A with Eritoran did not appear to confer any clear benefit to elective cardiac surgical patients.

4.5.2 Alkaline phosphatase

A physiological role for alkaline phosphatase was proposed in 1997 by Poelstra et al [48]. Alkaline phosphatase dephosphorylates and thereby detoxifies endotoxins (lipopolysaccharides) at physiological pH levels.
The phosphorylated lipid A moiety of endotoxin is a substrate for alkaline phosphatase, which enzymatically dephosphorylates the toxic lipid A part into monophosphoryl lipid A [49]. In the intestine alkaline phosphatase detoxifies endotoxines and prevents inflammation in response to gut microbiota [50].

Bovine intestinal alkaline phosphatase (bIAP) has been used in an animal model in sepsis and inflammatory bowel disease [51]. A randomized, double blind, placebo controlled study with bIAP in coronary artery bypass surgery (CABG) was performed by our group in 2006 [52]. In that study we found a significant TNFα response only in the placebo-treated group. In this group, next to TNFα, IL-6 and IL-8 were also increased. Such a TNFα response was not observed in the bIAP group, suggesting that there might be a role for bIAP in the attenuation of the inflammatory reaction post-CPB. We did not find any significant differences in clinical outcome, defined as postoperative complications and in-hospital mortality between the two groups.

5 Endotoxin adsorbers

5.1 Alteco® LPS adsorber

In 2009, Blomquist et al. [53] reported on a new endotoxin adsorber device called the Alteco® LPS adsorber. The adsorber consisted of porous polyethylene discs to which a specific polypeptide is bound that bind to the A moiety of endotoxin. The Adsorber was included in the bypass circuit between the arterial filter and the venous reservoir. The study was performed in 15 patients undergoing either elective CABG and/or valvular surgery with the use of CPB. In nine of these patients the LPS adsorber was included in the circuit. At several time points, blood samples were taken for endotoxin and different cytokines like TNFα, IL-6 and IL-1β. Endotoxin was found only in two patients (one in each group), with long cross clamp times. There were no significant differences in cytokine levels between both groups; therefore, the authors conluded that this adsorber device can be used safely in the bypass circuit, but that the intended effects of the adsorber could not be demonstrated. Recently the same adsorber has been used in a study performed by De Silva et al. [54]. In a prospective randomized controlled pilot trial, 17 patients were included with an expected CPB time of over 60 min. No significant differences were seen in endotoxin levels or cytokine levels between the adsorber and the control group.

5.2 Polymyxin B-immobilized hemoperfusion cartridge

Polymyxin B has both bacterial and anti-endotoxin capabilities. It can destroy bacterial outer membranes and bind endotoxin, thereby neutralizing its toxic effects [55]. Cartridges containing polymyxin B-immobilized fibers (Toramyxin PMX-F) are used in extracorporeal hemoperfusion to remove circulating endotoxin in patients with severe sepsis, with promising results [56]. In 2007 Ohki et al. [57] reported on an animal study in pigs. Ten pigs were divided into control and PMX groups undergoing normothermic CPB. The IL-8 level 2 h post CPB was significantly lower in the PMX treated group ($p < 0.05$). Cardiac and pulmonary functions, determined by measurement of cardiac output, left ventricular pressure and end-systolic pressure-volume ratio, were well preserved.

6 CPB related factors

6.1 Off pump versus on pump

In a review article by Raja and Berg [58] 19 randomized controlled trials comparing off-pump versus on-pump technique are described, focused on the systemic inflammatory response. They concluded that although off-pump CABG reduces the systemic inflammatory response, it does not abolish it completely. The inflammatory response during off-pump CABG is the result of the response to the surgical trauma, manipulation of the heart, pericardial suction and, other factors like anaesthesia.
In 2002 Aydin et al. compared the on- and off pump technique focusing on endotoxemia [59]. In 30 patients randomized between the on- and off pump technique, they demonstrated significantly lower endotoxin and lactate levels in the off-pump group and they concluded that this might lead to an improved recovery after off-pump CABG.

6.2 Pulsatile versus non-pulsatile flow

Watarida et al. [60] showed significantly lower levels of endotoxin during CPB using pulsatile flow as compared with nonpulsatile flow. An explanation for this might be the better splanchnic circulation during pulsatile flow leading to a decreased ischemia-induced translocation of bacteria in the portal circulation. Neuhof et al. [61] published similar results in 2001. In 48 patients randomized between pulsatile and nonpulsatile flow, they also demonstrated a significantly lower level of endotoxin when pulsatile flow was used during CPB. Both studies however did not show any differences in clinical outcome.

6.3 Hypothermia versus normothermia

In 2007, Rasmussen et al. [62] performed a randomized clinical study comparing the release of systemic mediators in normothermic and hypothermic (32 °C) CABG. They found no differences in mediator release between the two groups. Study limitations were, however, the small group size (n=30) and low risk patients, which may have affected study outcome.
In 1997 Gercekoglu et al. [63] studied endotoxin release during hypothermia. In 20 patients who underwent elective CABG they compared the endotoxin release between mild- (32-24 °C) versus deep (24-28 °C) hypothermia. They found significantly higher endotoxin levels in the group that underwent CABG with deep hypothermia as compared with the group in mild hypothermia. An explanation for this might be that hypothermia causes mucosal ischemia, and that during hypothermia the immune system and enzyme activities are depressed so that endotoxins are less efficiently detoxified by Kuppfer cells. However, both above mentioned studies showed no difference in clinical outcome between the different groups.

7 Conclusion

In this review we discussed the different strategies to reduce the endotoxin release in CABG patients with the use of CPB. Though many different strategies have been discussed in the literature, none of them described statistically significant clinical improvement when endotoxins were detoxified. Probably the studied population of mostly low risk CABG patients is a limiting factor that might explain this outcome.

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Bovine intestinal alkaline phosphatase reduces inflammation after induction of acute myocardial infarction in mice

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Abstract

Objective. There has been increasing evidence suggesting that lipopolysaccharide or endotoxin may be an important activator of the innate immune system after acute myocardial infarction. Bovine intestinal alkaline phosphatase reduces inflammation in several endotoxin mediated diseases by dephosphorylation of the lipid A moiety of lipopolysaccharide. The aim of this study was to investigate the effect of bovine intestinal alkaline phosphatase on reducing inflammation after acute myocardial infarction.

Methods and Results. Just before permanent ligation of the left anterior descending coronary (LAD) artery to induce acute myocardial infarction in Balb/c mice, bovine intestinal alkaline phosphatase (bIAP) was administrated intravenously. After 4 hours, mice were sacrificed and the inflammatory response was assessed. Acute myocardial infarction induced the production of different cytokines, which were measured in blood. Treatment with bovine intestinal alkaline phosphatase resulted in a significant reduction of the pro-inflammatory cytokines IL-6, IL-1β and the chymase mouse mast cell protease-1. No difference in the production of the anti-inflammatory cytokine IL-10 was observed between the control group and the bovine intestinal alkaline phosphatase treated group.

Conclusion. In a mouse model of permanent LAD coronary artery ligation, bIAP diminishes the pro-inflammatory responses but does not have an effect on the anti-inflammatory response in the acute phase after acute myocardial infarction.

1 Introduction

Lipopolysaccharide (LPS), an endotoxin present in the outer cell wall of Gram-negative bacteria, is a potent stimulator of the innate immune response. When entering the circulation, LPS binds to the lipopolysaccharide-binding protein (LBP) which interacts with CD14, MD2, and Toll-like receptor 4 (TLR4) to start a signaling cascade leading to cytokine production [1-4].

Cardiogenic shock is the leading cause of death among patients hospitalized with acute myocardial infarction (AMI). It is well known that AMI is associated with an increased systemic and local inflammatory response [5]. There is growing evidence suggesting that endotoxin is an important stimulus for this phenomenon. Decreased cardiac function reduces bowel perfusion, leading to hypoperfusion and ischemia of the intestinal mucosa. This results in increase of gut permeability, and subsequent translocation of endotoxin into the circulation [6;7]. Several studies with patients in heart failure as a result of cardiogenic shock, irrespective of etiology, have shown an increase of soluble CD14 (sCD14) in plasma, TLR4 expression on monocytes and increased levels of bacteria or endotoxin when compared to control groups [6;8-12]. Furthermore, elevated procalcitonin levels correlated to IL-6 levels have been described after acute myocardial infarction. Bacterial toxins are by far the most potent trigger for elevated procalcitonin levels [13]. Taken together, these data lead to suggest that endotoxin release is an important mediator in the observed inflammatory response after AMI.

There is increasing evidence that alkaline phosphatase is able to remove one phosphate group from the lipid A moiety of LPS, thereby dephosphorylating and detoxifying LPS [14;15]. In mice, infected with a lethal dose of Gram-negative bacteria, mortality was reduced after injection of human placental alkaline phosphatase (HPLAP) or bovine intestinal alkaline phosphatase (bIAP) [16;17]. A reduction in the inflammatory response induced by LPS could be observed in mice and piglets after treatment with HPLAP or bIAP [16;18]. Oral treatment of rats with LPS resulted in a prolonged endotoxemia after inhibition of endogenous intestinal alkaline phosphatase [19]. In addition, the potential effects of alkaline phosphatase on LPS-mediated diseases have been demonstrated in animal studies with polymicrobial sepsis. Cytokine response and neutrophil influx in secondary peritonitis in mice were attenuated by bIAP [20]. Hepatic and pulmonary injury after liver ischemia-reperfusion with partial resection was reduced in rats treated with bIAP when compared to control animals [21]. Studies performed by the group of Vincent et al. with bIAP administration to sheep, injected with an ultimately lethal dose of feces to mimic severe endotoxemia conditions, showed a decrease in IL-6 concentrations and a prolonged survival time [22]. In the study reported here, the left anterior descending (LAD) coronary artery ligation was used as a model to induce an AMI in mice. The primary endpoint was to examine the potential effect of bIAP on reducing the pro-inflammatory response principally depicted by IL-6 release in the acute phase after AMI by its ability to detoxify LPS. At the time point of peak IL-6 release complementary measurements of pro-inflammatory cytokines TNFα and IL-1β,

and anti-inflammatory cytokine IL-10 were performed. Prior to LAD ligation, bIAP was used as a prophylaxis by intravenous administration. The resulting systemic inflammatory response was investigated.

2 Methods

2.1 Induction of acute myocardial infarction

Animals used in the present study were treated in conformity with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, 1996). The study was approved by the animal ethics committee of the Faculty of Veterinary Medicine, Utrecht University. Specific pathogen free (SPF) female BALB/c mice (23-27 gram) were purchased from Charles River (Sulzfeld, Germany) and were acclimatized for 1 week under barrier conditions in filter-topped macrolon cages with drinking water and standard food pellets ad libitum. Mice were anaesthetized by inhalation of a mixture of O_2 air and 4% isoflurane, endotracheally intubated, and mechanically ventilated. The LAD coronary artery was exposed via a left thoractomy and double ligated with an 8.0 prolene suture, as described by Salto-Tellez et al [23]. To determine at which time point after AMI induction pro-inflammatory cytokine production could be detected, mice were sacrificed at 4, 6, and 24 hours respectively after AMI (n = 3 mice per time point) induction and blood was collected.

2.1 Bovine intestinal alkaline phosphatase

Clinical grade bovine intestinal alkaline phosphatase was obtained from Biozyme (Blaenavon, UK). One unit is defined as that amount of bIAP able to hydrolyse 1 μmole of p-nitrophenyl phosphate per minute using a Tris-glycin buffer at pH 9.6 at 25 ˚C. To examine the effect of bIAP, mice were divided into two groups: an AMI group treated with bIAP (n = 4) and an AMI control group (n = 4). BIAP was injected into the tail vein just before induction of anesthesia as a single intravenous dose of 5 IU in 100 μl phosphate buffered saline (PBS) (approximately 30-50 times above plasma levels). Control mice were injected with an equal volume of PBS. Mice were sacrificed and blood was collected. Heart, lung, liver and kidneys were removed and fixed in 4% para-formaldehyde in PBS.

2.2 Determination of alkaline phosphatase activity

Five μl of serum was incubated for 60 minutes at 37 ˚C with 200 μl assay mix containing incubation buffer (0.025 M glycin/NaOH, pH 9.6), p-nitrophenyl phosphate and $MgCl_2$ at final concentrations of 1.25 and 2 mM respectively. The end product p-nitrophenol was quantatively determined by measuring the extinction at 405 nm. Blood samples were centrifuged and serum was collected for determination of mouse IL-6. At the time point of peak IL-6 release complementary measurements of TNF-α, IL-1β and IL-10 protein were performed by commercially available ELISA kits according to the manufacturers' protocol (IL-6 and TNF-α from Biosource, Etten-

Leur, The Netherlands; IL-1β from R&D Systems, Abingdon, UK; and IL-10 from BD Biosciences, Erembodegem, Belgium). After AMI in mammals, mast cells are activated to release chymases. Activation of mast cells in mice can be measured by the release of the mouse mast cell protease-1 (mMCP-1) chymase. MMCP-1 ELISA was from Moredun (Midlothian, Scotland, UK) and performed according to the manufacturer's instructions.

2.3 Statistics

All data presented are mean ± SEM. Statistical analysis was performed using Student's *t*-test for unpaired data (GraphPad Prism). Values were considered significant when $p < 0.05$.

3 Results

3.1 Determination of the IL-6 response

In 9 mice it was determined at which time point after AMI in Balb/c mice IL-6 production could be detected. Before operation, IL-6 concentration was below detection limit (< 4 pg/ml) (Figure 1). Peak IL-6 serum levels were observed 4 hours after AMI. Elevated serum levels of IL-6 could still be detected 6 and 24 hours after AMI. Based on these results, mice were sacrificed 4 hours after AMI in the bIAP treatment experiments.

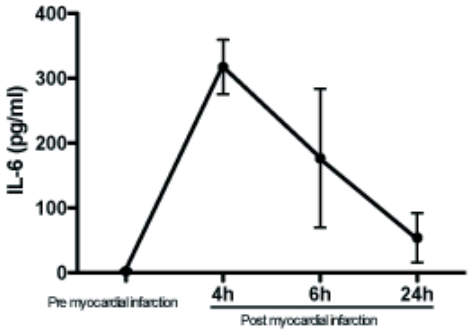

Figure 1 Production of the pro-inflammatory cytokine IL-6 after acute myocardial infarction. Mice were sacrificed at different time points and IL-6 production was determined ($n = 3$ per time point). Values are depicted as mean ± SEM.

3.2 Bovine alkaline phosphatase activity

Alkaline phosphatase activity was determined in serum samples by measuring hydrolysis of p-nitrophenyl phosphate by alkaline phosphatase. All mice that received bIAP had slightly elevated serum levels of alkaline phosphatase activity 4 hours after AMI compared to control mice ($p < 0.05$). The amount of alkaline phosphatase administered was about 30-50 times total plasma level. As reported

earlier by Beumer et al. [16] The total amount of administered bIAP falls within 10 minutes, thus the plasma level at timepoint of 4 hours represents a confirmation on successful intravenous administration of bIAP via the tail vein (Figure 2).

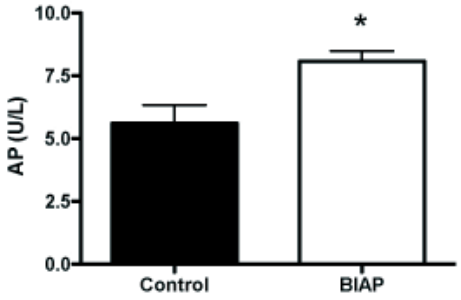

Figure 2 Alkaline phosphatase activity 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM (*n* = 4 per treatment group). * $p < 0.05$ versus control

3.3 Cytokine response

Before LAD coronary artery ligation, concentrations of the different cytokines were below detection levels. In contrast, 4 hours after AMI IL-6, IL-1β and IL-10 were excessively produced (Figure 3). TNF-α production could not be determined at this time-point. Administration of bIAP resulted in a significant reduction of the pro-inflammatory cytokines IL-6 and IL-1β as compared to controls. IL-6 levels were reduced by approximately 40% and IL-1β levels by approximately 30%. No difference in the anti-inflammatory cytokine IL-10 production could be observed between the control group and the bIAP-treated group.

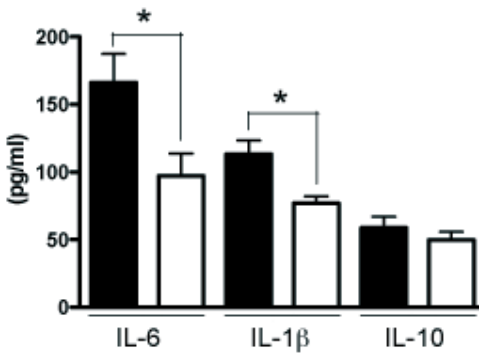

Figure 3 Effect of bIAP on the production of the pro-inflammatory cytokines IL-6 and IL-1β and on the anti-inflammatory cytokine IL-10 4 hours after acute myocardial infarction. Levels of IL-6, IL-1β, and IL-10 were determined using specific ELISA; (■) control mice and (□) bIAP-treated mice. Values are depicted as mean ± SEM (*n* = 4 per treatment group). * $p < 0.05$ versus control.

3.4 Mast cell activation

Serum levels of mMCP-1 were 14.7 ng/ml 4 hours after LAD coronary artery ligation. BIAP treatment reduced mMCP-1 levels in serum to 8.4 ng/ml (approximately 40%), implying a significant reduction in mast cell activation ($P < 0.05$) (Figure 4).

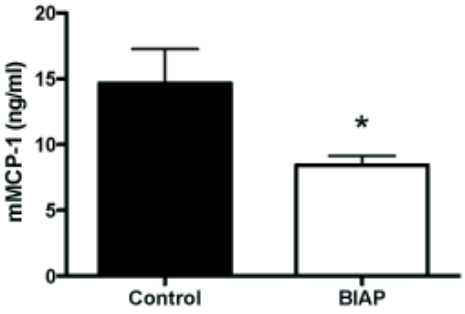

Figure 4 Effect of bIAP on the production of mMCP-1, 4 hours after acute myocardial infarction. Values are depicted as mean ± SEM ($n = 4$ per treatment group). * $p < 0.05$ versus control.

4 Discussion

Cardiogenic shock is the major cause of death in patients hospitalized with acute myocardial infarction (AMI) [24]. AMI results in intestinal hypoperfusion, which leads to increased gut permeability. Consequently, translocation of endotoxin into the circulation occurs. There has been growing evidence that presence of endotoxin is responsible for the observed systemic inflammatory response after AMI and that this may play an important role in the onset of cardiogenic shock [6;7;12;13]. Reducing inflammation after AMI has received little attention in research, and a specific pharmacologic treatment to reduce the inflammatory response after AMI has yet to be introduced.

To date, several studies have demonstrated the potential therapeutic effect of bIAP on LPS-mediated diseases, and it was therefore interesting to examine the ability of bIAP to reduce inflammation after AMI [20-22]. Therefore, Balb/c mice received an intravenous injection of bIAP just before AMI induction by permanent ligation of the LAD coronary artery.

Four hours after AMI, a significant reduction in the concentrations of the two most prominent pro-inflammatory cytokines present in serum in the acute phase after AMI being IL-6 and IL-1β, was observed when compared to non-bIAP treated controls. TNF-α concentration in serum, generally believed to be an early-onset pro-inflammatory cytokine, was below detection limit, suggesting that the chosen time point is not relevant to detect this cytokine in Balb/c mice after LAD coronary artery ligation. A reduction in pro-inflammatory cytokine production indicates a diminished systemic innate immune response, which may decrease myocardial dysfunction and reduce the development of cardiogenic shock after AMI [8]. It is

well known that inhibition of the complement-dependent inflammatory response, responsible for cellular alterations associated with irreversible myocardial injury, limits the extent of myocardial infarcts [25;26]. Thus, we performed a CH50 cell lysis assay to exclude the effect of bIAP on the alternative complement pathway. That assay demonstrated that high doses of bIAP (181 U/ml) resulted in inhibition of complement of 34%. In the low dose bIAP we used in the current study, no inhibition of complement could be measured (data not shown). BIAP treatment had no effect on IL-10 production. Since IL-10 is a potent anti-inflammatory cytokine, and several in vivo studies have shown its protective role in a variety of pathological states (e.g. colitis, hepatic ischemia/reperfusion and myocardial ischemia/reperfusion), a reduced production due to bIAP treatment would not be favorable [27-29]. Chymases are abundantly produced after AMI in mammals, and are known to be involved in the cleaving of angiotensin I to form angiotensin II [30;31]. The excessive formation of angiotensin II, which is observed in the acute phase after AMI, is arrhythmogenic, and several studies in different animal models have shown that decreasing angiotensin II formation by a specific chymase inhibitor contributes to a reduction in mortality rate in the acute phase after AMI [32;33]. Studies in rats revealed that production of the rat chymase MCP-2 (rMCP-2) is increased after stimulation of mast cells with LPS [34;35]. Given that bIAP has an effect on decreasing LPS toxicity, the influence of bIAP on the formation of the mouse chymase mMCP-1 was determined. In bIAP-treated mice, mMCP-1 production was significantly reduced by approximately 40% when compared to non-bIAP treated mice. This might result in a reduction of angiotensin II formation and consequently a decrease in arrhythmias, which may improve cardiac function and reduce cardiogenic shock complications [36]. Direct effects of bIAP on LPS detoxification could not be determined in this study. Since it is reported that bIAP is able to detoxify LPS through dephosphorylation of the lipid A moiety, the Limulus amoebocyte lysate (LAL) assay cannot be used as it is unable to make a discrimination between lipid A and monophosphoryl lipid A (MPLA) [37]. Therefore, decreased activation of the innate immune response because of bIAP administration could not be linked to decreased LPS levels in this study, and thus the direct effect of bIAP on LPS could not be assessed. Nonetheless, the specific activity of human placental alkaline phosphatase HPLAP and bIAP against an LPS insult has been undoubtedly demonstrated in vivo [16;18]. Furthermore, it has been demonstrated that alkaline phosphatase dephosphorylates and thereby detoxifies not only endotoxins but also extracellular nucleotides [38]. Alkaline phosphatase converts these nucleotides into non-inflammatory nucleosines [39]. Both endotoxins and nucleotides are potent inflammatory triggers and are sensed as 'stranger' or 'danger' signals to the innate immune system, and subsequent local and systemic inflammatory responses (SIRS) may result from the exposure to these pro-inflammatory signals [40;41].

The physiological anti-inflammatory role of alkaline phosphatase as an important key factor in inflammatory insults has recently been confirmed in a model in zebra fish [42] as well as in a rat-enterocolitis model [43].

In conclusion, a single intravenous dose of bIAP reduced the production of the chymase mMCP-1 by mast cells and diminished the systemic pro-inflammatory cytokine response in the acute phase after AMI. Therefore, it is proposed that bIAP might represent a novel therapeutic drug in attenuating the pro-inflammatory response after AMI, thereby reducing the incidence of cardiogenic shock complications.

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Anti-Inflammatory Effects of Alkaline Phosphatase in Coronary Artery Bypass Surgery with Cardiopulmonary Bypass

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Abstract

Laboratory and clinical data have implicated endotoxin as an important factor in the inflammatory response to cardiopulmonary bypass. Alkaline phosphatase prevents endotoxin-induced systemic inflammation in animals and humans. We assessed the effects of the administration of bovine intestinal alkaline phosphatase on surgical complications in patients undergoing coronary artery bypass grafting. In a double blind, randomized, placebo-controlled study, a total of 63 patients undergoing coronary artery bypass grafting were enrolled. Bovine intestinal alkaline phosphatase or placebo was administered as an intravenous bolus followed by continuous infusion for 36 hours. The primary endpoint was reduction of post-surgical inflammation. No significant safety concerns were identified. The overall inflammatory response to coronary artery bypass grafting with cardiopulmonary bypass was low in both placebo and bovine intestinal alkaline phosphatase patient group. Five patients in the placebo group displayed a significant TNFα response followed by an increase in plasma levels of IL-6 and IL-8. Such a TNFα response was not observed in the bovine intestinal alkaline phosphatase group, suggesting anti-inflammatory activity of bovine intestinal alkaline phosphatase. Other variables related to systemic inflammation showed no statistically significant differences. Bovine intestinal alkaline phosphatase can be administered safely in an attempt to reduce the inflammatory response in coronary artery bypass grafting patients with a low to intermediate EuroSCORE. The anti-inflammatory effects might be more pronounced in patients developing more fulminant postoperative inflammatory responses. This will be investigated in a further trial with inclusion of patients undergoing complicated cardiac surgery, demanding extended cardiopulmonary bypass and aortic cross clamp time. In this article some recent patents related to the field are also discussed.

1 Introduction

A significant portion of morbidity and mortality observed following cardiac surgery is due to an inflammatory response [1]. This inflammatory response is initiated by contact of heparinized blood and endothelial surfaces. This leads to an acute body's defence and thus activation of primary blood constituents like complement, neutrophils, monocytes and platelets. Other mediators of the inflammatory response are anaphylatoxins, cytokines, reactive oxidants and endotoxins. Endotoxin is produced by intestinal flora and is normally confined to the lumen of the intestine by a barrier of endovascular cells. During cardiopulmonary bypass (CPB) mesenteric hypoperfusion occurs which results in a loss of barrier function and as a consequence bacterial endotoxins may enter the systemic circulation [2;3]. The amount of endotoxin in the systemic circulation appears to be related to CPB time and also to cross clamp time [4]. An endotoxin molecule consists of four different parts: a lipid A moiety, an inner core, an outer core and an O-antigen. The lipid A moiety of the endotoxin molecule is composed of two phosphorylated glucosamine saccharides. The two phosphate groups attached to the saccharides are essential for the toxic activity of lipid A [5]. Alkaline phosphatase is an endogenous ecto-enzyme, widely expressed in many organs that are exposed directly or indirectly to the external environment, like the gastrointestinal tract and the lungs. Exposure of cells to endotoxin results in upregulation of alkaline phosphatase, indicating that alkaline phosphatase serves a role in the natural defense system against an endotoxin insult [6]. The phosphorylated lipid-A moiety of endotoxin is a substrate for alkaline phosphatase, which enzymatically dephosphorylates the toxic lipid-A part into monophosphoryl lipid-A, a non-inflammatory metabolite, and inorganic phosphate [6;7]. In the intestine alkaline phosphatase detoxifies endotoxines and prevents inflammation in response to gut microbiota [8]. Bovine intestinal alkaline phosphatase (bIAP) has been used in an animal model in sepsis and inflammatory bowel disease [9]. In a clinical study in severe sepsis patients continuous infusion of bIAP significantly improved their renal function [10;11]. Another composition comprising of anti-TNF and anti-IL-6 antibodies and polyclonal antibodies is described by Kink for the treatment of sepsis [12].
We hypothesized that modulating the host response to endotoxin by intravenous administration of alkaline phosphatase may prove to be an effective way of reducing the adverse post-operative inflammatory effects of cardiopulmonary bypass surgery. Furthermore, we hypothesized that a limited ability to neutralize endotoxins, measured by the amount of circulating anti-endotoxin antibodies (IgM EndoCAb) [13], may play a role in a poor outcome after cardiac surgery. Primary endpoint of this study was reduction of the post-surgical inflammatory reaction. Next to that we studied the effect of bIAP on post-surgical complications.

2 Materials and methods

In this double blind, placebo-controlled study, patients undergoing coronary artery bypass grafting (CABG) were randomized to receive either bovine intestinal alkaline phosphatase (bIAP) or matching placebo. The study was approved by the Institutional Review Board on the16th of March 2006. The study drug bIAP was manufactured by Biozyme ltd (Bleanavon, Wales, UK) and Alloksys Life Sciences B.V. (Bunnik, The Netherlands).

2.1 Patient selection

After written informed consent was obtained, male or non-pregnant female patients aged ≥ 18 and with a EuroSCORE ≥ 2 and ≤ 6, scheduled to undergo non-emergent coronary artery bypass grafting with the use of cardiopulmonary bypass, were enrolled. Exclusion criteria were: redo or emergency operations, baseline alkaline phosphatase levels > 100 IU/L, evidence of significant hepatic disease or levels of total bilirubine > 34 μmol/L, ALT > 120 U/L or AST > 135 U/L, history or signs of pre-operative infections, immunomodulating medication (i.e. steroids) or patients who were scheduled to receive 'stress dosis' of glucocorticoids, renal failure, creatinin > 177 μmol/L or patients with chronic renal insufficiency requiring dialysis, planned use of leucocyte depletion filtration, preoperative ventilatory support, Body Mass Index > 30, history of idiopathic thrombocytopenia and vegetarians, possibly intolerant of bovine proteins.

2.2 Schedule of assessments

Relevant medical history, concomitant medication and physical examination were obtained at baseline and throughout the study until postoperative day 30. Blood samples (haematological parameters, clinical chemistry, cytokines e.g. IL-6, IL-8 and TNFα, anti-endotoxin antibody) were collected at several time points before, during and after treatment to evaluate the primary efficacy endpoints. Clinical parameters like length of ICU stay, duration of ventilation and length of hospital stay were recorded. Adverse events were documented. All clinical laboratory measurements were performed in our hospital. Cytokine measurements based on the Luminex method [14] were performed at the National Institute of Health reference laboratory University Medical Centre, Wilhelmina Children's Hospital, Utrecht, the Netherlands.

2.3 Study drug administration, rationale for safety and randomization

The study drug bovine Intestinal Alkaline Phosphatase (bIAP) or matching placebo, a sterile solution for infusion containing no bIAP (content 1 ml) in a 2 ml vial in an aqueous buffer containing 20 mM Tris-HCl, 5 mM Magnesium Chloride, 0.1 mM

Zinc Chloride, pH 7.3, with 25 % glycerol and human serum albumin as stabilizer, was administered as an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour at a flow rate of 4 ml per hour for 36 hours in order to maintain supranormal levels of alkaline phosphatase in blood. A phase I bIAP study demonstrated that 72-hour continuous infusions of up to a total of 16.000-48.000 IU (at 80 kg bodyweight) of bIAP was safe and well tolerated. No immune incompatibility was found as evidenced by lack of induction of specific antibodies to bIAP over a period of 90 days after administration. No drug-related adverse events were reported [15]. The responsible trial pharmacist at the pharmacy department performed randomization of the study drugs. In the postoperative period routinely assayed alkaline phosphatase results were blinded by the clinical laboratory.

2.4 Anti-endotoxin levels

Based on the correlation between anti-endotoxin titers (EndoCab titers) and post surgery inflammation we evaluated the pre-surgical EndoCab titers at the end of the inclusion period. The patients were grouped on the basis of low (< 70 mU/mL), normal (70 -150 mU/mL) or high (>150 mU/mL) anti-endotoxin titers. Cytokine levels, clinical parameters and outcome were compared among the different groups.

2.5 CPB technique

After median sternotomy and preparation of the internal mammary artery, all patients received 3 mg/kg heparin (Leo Pharma, the Netherlands) intravenously. Because we used low dose (200 ml, 10000 KIU/mL) aprotinin (Bayer Health Care Pharmaceuticals) added to the prime in all patients we repeated heparin administration 1 mg/kg every hour during CPB, regardless of the ACT. The CPB circuit consisted of a Biomedicus BP80 centrifugal pump (Medtronic, Minneapolis, MN, USA), a membrane oxygenator (Sorin Srl. Avant, Mirandola, Italy or Medtronic Affinity, Minneapolis, MN, USA, or Gish Biomedical, Rancho Santa Margaria, California, USA), a custom made collapsible venous reservoir (Sorin Biomedica, Mirandola, Italy) and a D980 Avant dual chambered hard-shell venous cardiotomy reservoir (Sorin Srl., Mirandola, Italy). Priming fluid consisted of 800 ml NaCl 0,9%, 500 ml Voluven® (Fresenius Kabi, the Netherlands), 200 ml Mannitol 20% (Baxter Health Care, the Netherlands), 200 ml Aprotinin 10000 KIU/mL, 25 ml NaHCO3 8,4% and Heparin 7500 IU. Normothermic cardiopulmonary bypass was applied in all patients. For myocardial protection, depending on the surgeon's preference, either warm blood cardioplegia or st. Thomas cold cristalloid cardioplegia was used. At the end of cardiopulmonary bypass heparin was neutralized with protamine chloride (Valeant Pharmaceuticals, the Netherlands).

2.6 Statistical analysis

Evaluation was performed with help of the SAS® System (Software Release 9.13). Data were checked for completeness and a second plausibility check was performed. The Wilcoxon signed rank test was used to compare continuous variables of two groups, the Pearson's chi-square test was used to investigate the frequency (percentage) to parameters, and a probability of $p<0.05$ was considered to be statistically significant. However, apart from the primary endpoint (frequency of major pro-inflammatory reaction) all p-values given are descriptive only.

3 Results

3.1 Patients and procedures

A total of 63 patients was enrolled in this study. No significant safety concerns were identified. The patients' clinical data are listed in Table 1. In the bIAP group BMI was significantly higher than in the placebo group. No statistical significant differences in other demographic data were observed. Mean EuroSCORE was 3.6 and 3.7 for bIAP and placebo treated group respectively. Analysis of the subpopulations of patients per EuroSCORE showed a notable difference in their pro-inflammatory peak values between EuroSCORE 2 and EuroSCORE 4. Peak IL-6 values for bIAP were 40% less compared to placebo peak IL-6 levels. The two groups were similar with regard to the number of grafts, CPB- and cross clamp time and type of cardioplegia used. In both groups, one patient underwent concomitant pulmonary vein isolation. There were no statistical significant differences in postoperative length of stay and postoperative complications.

	bIAP (n=32)	**placebo** (n=31)	**p**
Preoperative data			
Age (y)	71.4 ± 4.2	70.2 ± 6.8	0.495
Male	27	28	0.478
BMI (kg/m²)	27.4 ± 3.5	25.7 ± 2.7	0.037
Euroscore	3.63 ± 1.24	3.68 ± 1.42	0.682
EF < 30% (%)	3	6	0.535
Hx of COPD (%)	3.1	12.9	0.151
Hx of diabetes (%)	18.8	9.7	0.304
Preop serum creatinin (µmol/L)	90.6 ± 20.1	95.5 ± 17.5	0.153
Operative data			
Number of gafts	3.8	3.5	0.078
Total duration of surgery (hours)	2.95 ± 1.08	2.76 ± 0.58	0.874
CPB duration (min)	72.2 ± 41.2	61.5 ± 22.3	0.257
Cross clamp duration (min)	49.4 ± 27.3	44.7 ± 16.8	0.710
Aprotinin (1000 KIU/mL)	32	31	ns
Use of cell saver (%)	37.5	48.4	0.374
Warm blood cardioplegia (%)	71.9	77.4	0.613
St. Thomas cold cristalloid cardioplegia (%)	28.1	22.6	0.613
Normothermia (%)	100	100	ns
Concomitant PVISO	1	1	ns
Postoperative data			
Intensive care length of stay (hours)	18.3 ± 1.5	16.5 ± 1.8	0.146
Hospital length of stay (days)	6.1 ± 2.4	6.2 ± 2.8	0.847
Hospital readmision (%)	0	6.4	0.144
30 day mortality (%)	3.1	3.2	ns
Postoperative atrial fibrillation (%)	28.1	38.7	0.373
Postoperative infections (%)	9.6	9.6	ns
New stroke (%)	0	3.2	0.306

Table 1 Clinical data

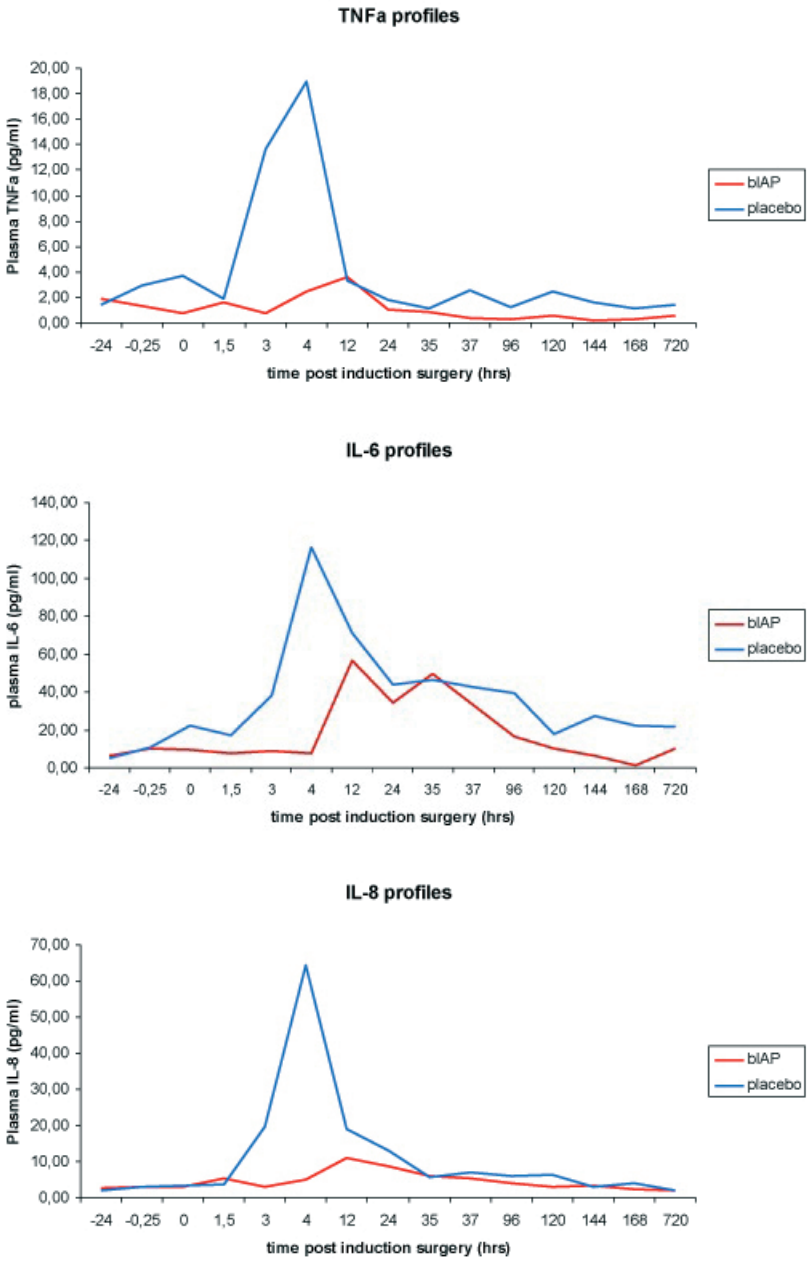

Figure 1 Plasma levels of TNFα, IL-6 and IL-8 in all patients

3.2 Cytokine levels

In the placebo group 5 patients displayed a significant TNFα response. The mean peak level of TNFα in these 5 patients was 108.1 ± 205.9 pg/ml and was observed at 4 hours post induction of surgery. This TNFα response was followed by an increase in plasma

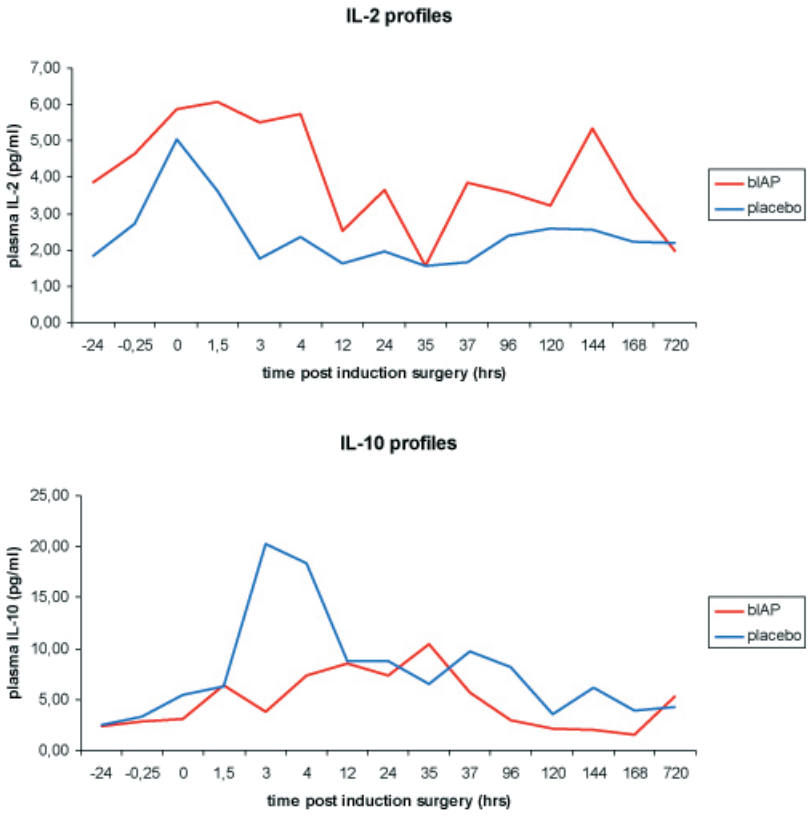

Figure 2 Plasma levels of anti-inflammatory cytokines IL-2 and IL-10 in all patients

levels of IL-6 (682.6 ± 965.0 pg/ml) and IL-8 (641.9 ± 836.7 pg/ml). In contrast, in these 5 patients the anti-inflammatory cytokines IL-10 (56.58 ± 44.03), IL-2 (0.15 ± 0.12 pg/ml) and IL-4 (0.12 ± 0.05 pg/ml) did not show any response. Such a post-surgical TNFα response was not observed in the bIAP group ($p<0.02$). Figure 1 shows the plasma levels of IL-6, IL-8 and TNFα of all patients in the bIAP and placebo group.

Figure 2 shows the anti-inflammatory cytokines IL-2 and IL-10 of all patients. Interestingly, the overall inflammatory response was low both in the bIAP and the placebo group. However, the peak level for cytokine IL-6 was observed at 4 hours post induction of surgery and was 64.4 ± 133.7 pg/ml in the bIAP group and 140.3 ± 428.2 pg/ml in the placebo group (p = ns). At 4 hours post induction of surgery also the peak level for cytokine IL-8 was observed and was 27.2 ± 35.8 pg/ml in the bIAP group and 117.3 ± 385.1 pg/ml in the placebo group (p = ns). In the total study population, we did not observe statistically significant differences in haematological data outcome, especially inflammatory parameters like white blood cell count and CRP levels, between the bIAP and the placebo group.

3.3 Anti-endotoxin levels

There were no statistically significant differences in distribution of bIAP and placebo between the low endocab level (< 70 mU/ml) group and intermediate endocab level (between 70 and 150 mU/ml) group. In the high endocab level (> 150 mU/ml) group one patient was treated with placebo. This patient hardly showed any reaction of IL-6 and IL-8, 9.1 and 4.6 pg/ml respectively (Table 2). Although there is an obvious trend that low endocab levels are followed by a higher cytokine level, this can only be explained by the 5 patients in the placebo group that showed a significant TNFα response.

	bIAP	**placebo**
Endocab level < 70	**n=30**	**n=26**
IL-6	99.2 ± 162.81	170 ± 326.53
IL-8	39.7 ± 50.04	157 ± 418.83
Endocab level 70-150	**n=2**	**n=3**
IL-6	184 ± 238.71	40.3 ± 38.46
IL-8	26.8 ± 18.75	8.42 ± 6.02
Endocab level > 150	**n=0**	**n=1**
IL-6		9.09
IL-8		4.59

Table 2 Endocab levels and cytokines

4 Discussion

A significant portion of morbidity and mortality observed following cardiac surgery has been proposed to be due to an inflammatory response induced by endotoxin [2-4]. Endotoxin is produced by intestinal flora and is normally confined to the lumen of the intestine. CABG is associated with increased LPS translocation from the intestine [16], and is recognized to be a major stimulus for the development of the systemic inflammatory response syndrome. Hence, Brands *et al*. describe the use of alkaline phosphatase for the prophylaxis or treatment of LPS mediated diseases [17]. However, the reported circulating endotoxin concentrations and the duration of exposure to endotoxin and subsequent pro-inflammatory response vary among different articles and may depend on methodology used at the clinical site [3;18]. Once in the circulation, excess of endotoxin levels induces inflammatory cytokines provoking an inflammatory response and ultimately activation of e.g. neutrophils. Neutrophil activation is associated with organ hypoperfusion resulting in multiorgan dysfunction including cardiovascular dysfunction, kidney dysfunction and acute respiratory distress syndrome [1;19]. Reduction of endotoxin levels may result in an attenuation of the postoperative inflammatory response.

Alkaline phosphatase has been shown to be able to detoxify endotoxin [6-8]. In recent animal studies promising therapeutic effects of alkaline phosphatase were described [9;20]. In these preclinical studies it was demonstrated that during an inflammatory insult endogenous alkaline phosphatase levels in plasma are reduced. In our phase II trial, infusion of bIAP was started prior to surgery and continued up to 36 hours post induction of surgery in order to supplement for endogenous alkaline phosphatase.

Interestingly, in the present study the overall inflammatory response appeared to be low in both the bIAP and the placebo group. In several reported clinical trials higher levels of pro-inflammatory cytokines are reported [13;21].
The improvement of surgical techniques and of the CPB circuit over the last few years may have resulted in lower inflammatory reactions and better overall clinical outcome. Endotoxin release followed by an increase in pro-inflammatory cytokines is related to CPB time and aortic cross clamp time [4;22]. In our study mean CPB time and aortic cross clamp time were possibly too short to lead to a significant increase in pro-inflammatory parameters. However, in the placebo group, 5 patients showed a significant TNFα response with a subsequent rise in IL-6 and IL-8, but without any effect on the levels of the anti-inflammatory cytokines IL-2, IL-4 and IL-10.
In contrast, no patient in the bIAP group responded in a similar way, strongly suggesting that there might be a role for bIAP in CABG patients in reducing the post-surgical inflammatory response and confirming pre-clinical reported data on the proposed physiological function of alkaline phosphatase [6;9;20].
Since normothermia was applied in all our patients, this may be another explanation for the low inflammatory response. It is described that hypothermia leads to significantly higher levels of endotoxin with increasing levels of endotoxin during and after deep hypothermia [23].

The low occurrence of overall inflammatory response might also be explained by the included patient population with a EuroSCORE from 2-6. The EuroSCORE has a direct correlation with postsurgical chance of morbidity and mortality [24] and it may also be linked to the immunological status of the patient. Analysis of the subpopulations of patients per EuroSCORE showed a notable difference in peak values of their pro-inflammatory peak values between EuroSCORE 2 and EuroSCORE 4. Also peak IL-6 values for bIAP were 40% less than placebo peak IL-6 levels. Further analysis among the different subgroups was not performed because of the small number of patients in the different subgroups.

Low preoperative serum Endocab level is a significant predictor of long-term mortality independent of other known risk factors [13;25]. In our study we noted in the majority of patients a low preoperative serum endocab level. We did not find any

statistical differences between the bIAP and placebo group with regard to Endocab levels and clinical outcome. An obvious trend is noticed, indicating that low Endocab levels are associated with a higher cytokine level.

Importantly, this study was powered to include 100 patients, then perform an interim analysis and if necessary include 50 patients more. All patients were treated with low dose aprotinin in the prime fluid of the CPB circuit. However, since the use of aprotinin was suspended in November 2007 by the FDA, based on preliminary results of the BART trial [26], we were forced to end our study preliminarily, resulting in two smaller groups of patients than anticipated.
Nevertheless the administration of aprotinin was primary focused on the reduction of postoperative blood loss. But with regard to the use of aprotinin in our patients it is also important to point out that aprotinin might not only play a role in the reduction of bloodloss but also in the reduction of the inflammatory response [27]. This, however, was not confirmed in recent studies, albeit carried out under different conditions, which may underline the impact of the methodology applied [28;29]. Another example is described by Ladner *et al*. They provide methods for reducing blood loss and systemic inflammatory response after cardiopulmonary bypass [30].

5 Current & future developments

In this phase II study we show that bIAP can be administered safely in CABG patients with a low to intermediate EuroSCORE. bIAP might be effective in reducing overall post surgical inflammation and ischemic reperfusion damage. The anti-inflammatory effect of bIAP might be more pronounced in patients developing more fulminant postoperative inflammatory responses. This will be investigated in a further trial with inclusion of patients undergoing complicated cardiac surgery, demanding extended CPB and aortic cross clamp time.

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Chapter 5

Prophylactic treatment with alkaline phosphatase in cardiac surgery induces endogenous alkaline phosphatase release

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Abstract

Purpose: Laboratory and clinical data have implicated endotoxin as an important factor in the inflammatory response to cardiopulmonary bypass. We assessed the effects of the administration of bovine intestinal alkaline phosphatase, an endotoxin detoxifier, on alkaline phosphatase levels in patients undergoing coronary artery bypass grafting.

Methods: A total of 63 patients undergoing coronary artery bypass grafting was enrolled and prospectively randomized. Bovine intestinal alkaline phosphatase (n=32) or placebo (n=31) was administered as an intravenous bolus followed by continuous infusion for 36 hours. The primary endpoint was to evaluate alkaline phosphatase levels in both groups and to find out if administration of bIAP to patients undergoing CABG would lead to endogenous alkaline phosphatase release.

Results: No significant adverse effects were identified in both groups. In all the 32 patients of the bIAP treated group, we found an initial rise of plasma alkaline phosphatase levels due to bolus administration (464.27 ± 176.17 IU/L). A significant increase of plasma alkaline phosphatase at 4-6 hours postoperatively was observed (354.97 ± 95.00 IU/L) as well. Using LHA inhibition, it was shown that this second peak was caused by the generation of Tissue Non Specific Alkaline Phosphatase (TNSALP-type alkaline phosphatase).

Conclusions: Intravenous bolus administration plus 8 hours continuous infusion of alkaline phosphatase in patients undergoing coronary artery bypass grafting with cardiopulmonary bypass results in endogenous alkaline phosphatase release. This endogenous alkaline phosphatase may play a role in the immune defence system.

Introduction

Alkaline phosphatase is a ubiquitous endogenous ecto-enzyme in the human body. This ectophosphatase is widely expressed in many organs that are exposed directly or indirectly to the external environment, like the gastrointestinal tract and the lungs. A physiological role for alkaline phosphatase was proposed in 1997 by Poelstra et al [1]. Alkaline phosphatase dephosphorylates and thereby detoxifies not only endotoxins (lipopolysaccharides) but also extracellular nucleotides [2;3]. Alkaline phosphatase converts these nucleotides into non-inflammatory nucleosines [4]. Both endotoxins and nucleotides are potent inflammatory triggers and are sensed as 'stranger' or 'danger' signals to the innate immune system, and subsequent local and systemic inflammatory responses (SIRS) may result from the exposure to these pro-inflammatory signals [5].

During cardiopulmonary bypass (CPB), hypoperfusion of the gut may result in a loss of barrier function and as a consequence bacterial endotoxins, normally confined to the lumen of the intestine by a barrier of endovascular cells, may enter the systemic circulation [6;7]. The amount of endotoxin release into the circulation seems to be related to the duration of cross clamping time and CPB time. In this regard, endotoxin release has been recognized as an important factor in the inflammatory response following CPB [8].

In previous animal studies promising therapeutic effects in reducing the inflammatory response with the use of intravenous alkaline phosphatase are shown [9-11]. In a clinical study in severe sepsis patients continuous infusion of calf-intestinal alkaline phosphatase significantly improved renal function [12;13].

It is known that the pro-and anti-inflammatory response can be manipulated by positive feedback mechanisms. However, it is not known whether alkaline phosphatase infusion can induce such a positive feedback. In an earlier report, we studied the possible beneficial effect of bovine intestinal alkaline phosphatase (bIAP) on inflammatory parameters post CPB in a randomized, double blind, placebo controlled study with bIAP in patients undergoing elective coronary artery bypass grafting (CABG) with the use of CPB (the APPIRED study). [14] In that phase of the study, we investigated the effect of bIAP on inflammatory parameters after CPB and the possible clinical effects of bIAP on the prevention of systemic inflammatory response syndrome (SIRS). In the present sub-study, we investigated the levels of alkaline phosphatase in patients undergoing CABG with CPB, treated with either placebo or bIAP. We hypothesized that administration of bIAP would increase endogenous alkaline phosphatase release in patients undergoing CABG with the use of CPB.

Materials and Methods

In a double blind, placebo-controlled study, 63 patients undergoing elective CABG with the use of CPB were randomized to receive either bIAP (n=32) or matching placebo (n=31). The study was approved by the Institutional Review Board. An

informed consent was obtained from each participant. The study drug bIAP was manufactured by Biozyme ltd (Bleanavon, Wales, UK) and Alloksys Life Sciences B.V. (Bunnik, The Netherlands). The placebo also consisted of 1 ml sterile aqueous solution for infusion in an aqueous buffer containing 20 mM Tris-HCl, 5 mM Magnesium Chloride, 0.1 mM Zinc Chloride, pH 7.3, with 25 % glycerol containing no bIAP. The study drug bovine Intestinal Alkaline Phosphatase (bIAP) or matching placebo was administered as an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram per hour at a flow rate of 4 ml per hour for 36 hours in order to maintain supranormal levels of alkaline phosphatase in blood. A phase I bIAP study demonstrated that 72-hour continuous infusions of up to a total of 16.000-48.000 IU (at 80 kg bodyweight) of bIAP was safe and well tolerated. No immune incompatibility was found as evidenced by lack of induction of specific antibodies to bIAP over a period of 90 days after administration. No drug-related adverse events were reported [15].
The priming fluid of the CPB consisted of 800 ml NaCl 0,9%, 500 ml Voluven® (Fresenius Kabi, the Netherlands), 200 ml Mannitol 20% (Baxter Health Care, the Netherlands), 200 ml Aprotinin 1000 KIU/mL, 25 ml NaHCO3 8,4% and Heparin 7500 IU.
Blood samples (haematological parameters, clinical chemistry, cytokines e.g. IL-6, IL-8 and TNFα, anti-endotoxin antibody) were collected at several time points before, during and after surgery (24 and 0.25 hr before induction of anaesthesia and 0.25 hr, 1.5 hr, 3 hr, 4 hr, 12 hr, 24 hr, 35, 37 and 96 hr after induction of surgery, pointed out as visitnumbers 1-11). Furthermore, clinical parameters like length of ICU stay, duration of ventilation and length of hospital stay were recorded. An extended description of the materials and methods has been described earlier[14].
The primary endpoint of the present sub-study was to evaluate alkaline phosphatase levels in both groups and to find out if administration of bIAP to patients undergoing CABG would lead to endogenous alkaline phosphatase release.

Alkaline phoshatase measurement

Alkaline phosphatase was measured using a PNPP (p-nitrophenol phosphate) kinetic assay [16]. Samples were defrosted and warmed gradually to 21 degrees Celsius. 200 µliter of a serum sample was mixed with 1 ml of PNPP-substrate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and $MgCl_2$ (final concentration 2 mM) in a Tris-glycin buffer at pH 9.6. Samples were measured kinetically at 405nm on Biorad Smartspec photo spectrometer for 60 seconds with intervals of 20 seconds.

L-homo arginine (LHA) inhibition

L-homo arginine (LHA) (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used as a tissue non-specific alkaline phosphatase (TNSALP) inhibitor, to

investigate the origin of the endogenous alkaline phosphatase. LHA is supposed to have minimal influence on bIAP, but inhibits tissue non-specific alkaline phosphatase activity [17]. BIAP was diluted to a concentration of 0.08 U/L and 100 µL was added to a cuvette. 5mM LHA (final concentration) was added and this was gently mixed and incubated at room temperature for 5 minutes. Next a PNPP Kinetic assay was carried out similarly as described above, but now measurements lasted 180 seconds with intervals of 20 seconds. For pharmacokinetic purposes alkaline phosphatase levels in human blood plasma samples were measured at the Catharina Hospital Laboratory by routine clinical chemistry methods (Cobas-Bio centrifugal analyser, Roche Diagnostics, Switzerland).

Statistical analysis

When applicable, evaluation was performed with help of the SAS System (Software Release 9.13). Data were checked for completeness and a second plausibility check was performed. The Wilcoxon signed rank test was used to compare continuous variables, the Pearson's chi-square test was used to investigate the frequency (percentage) to parameters, and a probability of $p<0.05$ was considered to be statistically significant.

Results

A total of 63 patients (bIAP n=32, placebo n=31) was enrolled in this study. No significant safety concerns were identified. Baseline characteristics are listed in Table 1. Beside a significantly higher BMI in the bIAP treated group, no statistical significant differences in demographic data were observed.

An evident inflammatory response was only observed in 5 patients of the placebo group. In these 5 patients, we observed a fulminant TNFα response (mean peak level 108.1 pg/ml) at 3 to 4 hours post start of surgery. This TNFα response was followed by an increase in plasma levels of IL-6 and IL-8 (mean peak levels of 682.6 pg/ml and 641.9 pg/ml respectively). Such a TNFα response was not observed in the bIAP treated group ($p < 0.02$). The overall inflammatory response as deduced from cytokine levels, C reactive protein (CRP), AST and ALT was low both in the bIAP treated group and the placebo group. No significant differences in peri-operative complications were found between both groups.

Postoperative plasma Alkaline Phosphatase levels

Preoperative levels of alkaline phosphatase were 70.03 ± 17.12 IU/L in the bIAP treated group, and 70.50 ± 15.63 IU/L in the placebo treated group (p = ns). In all 31 patients of the placebo treated group, we found a reduction of plasma alkaline phosphatase levels within 2 hours after start of surgery (34.89 ± 9.59 IU/L). This reduction in plasma alkaline phosphatase levels was followed by normalisation of this level after 24 hours.

	bIAP (n=32)	**placebo** (n=31)	**p**
Preoperative data			
Age (y)	71.4 ± 4.2	70.2 ± 6.8	0.495
Male, n	27	28	0.478
BMI (kg/m²)	27.4 ± 3.5	25.7 ± 2.7	0.037
Euroscore (additive)	3.63 ± 1.24	3.68 ± 1.42	0.682
EF < 30%, n	3	6	0.535
COPD, n	1 (3.1)	4 (12.9)	0.151
Diabetes, n	6 (18.8)	3 (9.7)	0.304
Preop serum creatinin (µmol/L)	90.6 ± 20.1	95.5 ± 17.5	0.153
Operative data			
Number of gafts,	3.8 ± 1.1	3.5 ± 0.9	0.078
Total duration of surgery (hours)	2.95 ± 1.08	2.76 ± 0.58	0.874
CPB duration (min)	72.2 ± 41.2	61.5 ± 22.3	0.257
Cross clamp duration (min)	49.4 ± 27.3	44.7 ± 16.8	0.710
Aprotinin(1000 KIU/mL), n	32	31	ns
Use of cell saver, n	12 (37.5)	15 (48.4)	0.374
Warm blood cardioplegia, n	23 (71.9)	24 (77.4)	0.613
St. Thomas cold crystalloid cardioplegia, n	9 (28.1)	7 (22.6)	0.613
Normothermia, n	32 (100)	31 (100)	ns
Concomitant PVISO	1	1	ns
Postoperative data			
Intensive care length of stay (hours)	18.3 ± 1.5	16.5 ± 1.8	0.146
Hospital length of stay (days)	6.1 ± 2.4	6.2 ± 2.8	0.847
Hospital readmission, n	0 (0)	2 (6.4)	0.144
30 day mortality, n	1 (3.1)	1 (3.2)	ns
Postoperative atrial fibrillation, n	9 (28.1)	12 (38.7)	0.373
Postoperative infections, n	3 (9.6)	3 (9.6)	ns
New-onset stroke, n	0 (0)	1 (3.2)	0.306

Table 1 Baseline characteristics

Data are expressed as mean ± SD or numbers (%) unless otherwise mentioned

BMI= body mass index; COPD= chronic obstructive pulmonary disease;

CPB= cardiopulmonary bypass; EF= ejection fraction;

PVISO= pulmonary vein isolation

In all the 32 patients of the bIAP treated group we found an initial rise of plasma alkaline phosphatase levels due to bolus administration (464.27 ± 176.17 IU/L). Next to the initial rise a significant increase of plasma alkaline phosphatase at 4-6 hours postoperatively was observed (354.97 ± 95.00 IU/L) (Figure 1).

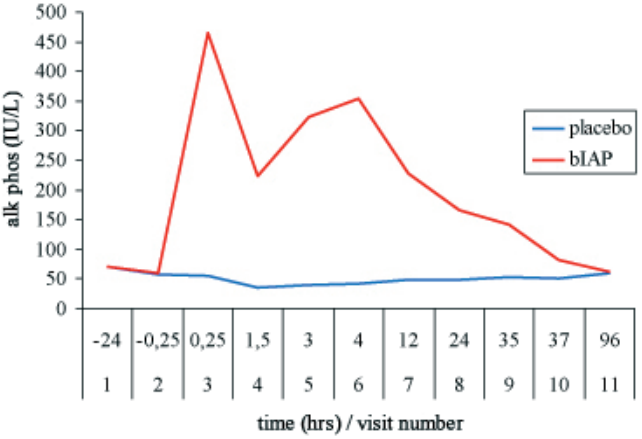

Figure 1 Alkaline phosphatase levels at different time points perioperatively. Visit numbers correspond to the different time points.

We used LHA to inhibit the second peak of alkaline phosphatase with LHA (Figure 2), suggesting that this second peak is caused by the generation of Tissue Non Specific Alkaline Phosphatase (TNSALP-type alkaline phosphatase) [17].

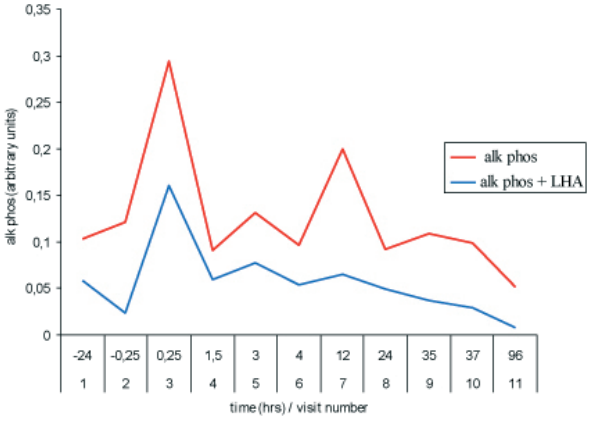

Figure 2 Alkaline phosphatase levels at different time points perioperatively either with or without LHA.

Through isoenzyme analysis it was excluded that this postoperative rise of plasma alkaline phosphatase could be attributed to rise of bone type alkaline phosphatase (Table 2).

visit number	total AP (IU/L)	bone AP (IU/L)
2	69.3 ± 21.2	19.4 ± 6.5
3	475 ± 183.8	19 ± 6.2
4	184 ± 33.2	11 ± 3.9
5	282 ± 44.8	12 ± 3.4
6	372 ± 43.8	15.4 ± 5.3
7	226 ± 39.5	16.8 ± 6.7
8	136 ± 63.6	17.4 ± 3.7

Table 2 Total alkaline phosphatase levels and bone type alkaline phosphatase levels

The mean value of serum bilirubin level in the placebo group increased from 10.87 ± 6.66 μmol/L preoperatively to 13.28 ± 6.13 μmol/L 24 hours postoperatively. In the bIAP group, this value increased significantly from 11.06 ± 5.36 μmol/L preoperatively to 16.11 ± 8.04 μmol/L 24 hours postoperatively.

Discussion

This prospective study demonstrates that administration of bIAP in patients undergoing CABG with the use of CPB leads to the induction of endogenous alkaline phosphatase. This may lead to augmentation of the anti-inflammatory effect of biAP in patients undergoing CABG with the use of CBP.

During CABG with the use of CPB, increased endotoxin translocation from the intestine occurs [18;19]. Moreover, it has been demonstrated that depending on CPB time and cross clamp time ischemic insults occur followed by a local rise in nucleotides. Both ischemia-reperfusion mediated endotoxin and extra-cellular released nucleotides are potent pro-inflammatory triggers and are a substrate for both supplemental and endogenous alkaline phosphatase. Normally LPS travels with chime and is taken up by both Kupffer cells and hepatocytes. This LPS is proposed to be predominantly detoxified through the activity of intestinal type alkaline phosphatase and systemic available alkaline phosphatase. In the absence of sufficient reactive alkaline phosphatase its endotoxin clearance function may be suboptimal, resulting in further aggravation of endotoxin-mediated inflammatory effects. That is the reason why we supplemented bovine alkaline phosphatase in our study to combat endotoxin-induced inflammation in CABG with the use of CPB.

An interesting finding in this study was the difference in alkaline phosphatase kinetics in plasma between the bIAP and the placebo treated group. In placebo treated patients a reduction of plasma alkaline phosphatase levels was measured 2 hours postoperatively. Normalised plasma levels were measured after 24 hours

postoperatively. Reduction of plasma alkaline phosphatase levels after endotoxin administration levels was noted previously by Verweij et al. in animal studies [20]. Kupffer cells may function to clear the alkaline phosphatase-LPS conjugates from the circulation, thereby reducing the total alkaline phosphatase levels [2;20]. This is also demonstrated in our study in the placebo group, having an initial reduction of plasma alkaline phosphatase. CABG with the use of CPB leads to a significant degree of hemodilution [21]. In the APPIRED study[14], the mean hematocrit value dropped to a mean of 0.28 L/L. The reduction of alkaline phosphatase can be partially due to this hemodilution of approximately 40%. However, the reduction in alkaline phosphatase exceeds this 40% and thus a real clearance of alkaline phosphatase-LPS conjugates from the circulation must occur in our studied CABG population. This could be attributed in part to clearence of LPS conjugates from the circulation by Kupffer cells. In the bIAP treated group, an initial rise in alkaline phosphatase plasma level due to the bolus administration was observed. The kinetic profile of this plasma alkaline phosphatase level was compatible with the administered alkaline phosphatase with a physical half-life of about 10 minutes [9]. Next to the initial increase in alkaline phosphatase level a significant increase of plasma alkaline phosphatase at 4-6 hours postoperatively was observed. This second peak of alkaline phosphatase is not representing the intravenously administered bIAP, because this peak represents a much larger amount of systemic circulating alkaline phosphatase than the administered bIAP dose, which is calculated to give a doubling of normal plasma alkaline phosphatase levels during the time of perfusion.

We sought to determine the origin of this endogenous released alkaline phosphatase in the bIAP treated group and also to explain the fact that only a bolus bIAP followed by continuous infusion leads to this endogenous alkaline phosphatase release.

This endogenous alkaline phosphatase is inhibited by L-homoarginine, known as an inhibitor of tissue non-specific alkaline phosphatase and thus likely represents tissue non-specific alkaline phosphatase, with a physical half life of about 20 hours [17]. As judged from the post-surgical amount of this tissue non-specific alkaline phosphatase circulating, the most likely source is liver type alkaline phosphatase, since it was demonstrated that the other abundant source being bone type alkaline phosphatase was not elevated. Taking here also the amount of hemodilution into account, it was interesting to find that also for hemodilution corrected bilirubin levels were elevated in this group which amplifies our idea of this endogenous alkaline phosphatase being liver type alkaline phosphatase.

This endogenous alkaline phosphatase release in patients undergoing cardiac surgery with the use of CPB, noted only in patients treated with a bIAP bolus followed by continuous infusion, is a unique finding that to our knowledge has not been described before. Pickkers et al. [12] reported on the pharmacology of exogenously administered alkaline phosphatase in healthy volunteers and severe sepsis patients. They also used a dosing schedule with a bolus alkaline phosphatase followed by a continuous infusion in both the healthy volunteer group and the severe sepsis group.

Although the total amount of administered alkaline phosphatase was higher in their patients, no endogenous alkaline phosphatase release was found in their group. Whether the use of CPB in our population is cause of different results needs to be further investigated. Tuin et al. demonstrated in vitro that LPS exposure to liver slices in situ results in increased mRNA for alkaline phosphatase expression with kinetics that are compatible with de novo synthesis [22].

We hypothesize that alkaline phosphatase prophylaxis improves the defence mechanism against a new inflammatory insult by triggering the release of sustainable alkaline phosphatase in the circulation possibly as a consequence of de-novo synthesis.

The surprising implication of this finding may have significant consequences. Alkaline phosphatase may act like an acute phase protein, where high levels of physiological active alkaline phosphatase have a protective anti-inflammatory effect. The preoperative plasma levels may predict clinical outcome in acute inflammation in a manner similar to that reported for high plasma anti-endotoxin antibody levels [23;24]. Patients might thus be protected by pre-treatment with physiological active alkaline phosphatase which will elevate their endogenous physiological levels. However, and as mentioned above, the mechanism of this postoperative rise of endogenous alkaline phosphatase observed in our study has yet to be elucidated and further studies are needed to investigate if this finding will have clinical advantage for CABG patients.

Conclusion

Intravenous bolus administration plus continuous infusion of alkaline phosphatase in patients undergoing coronary artery bypass grafting results in a subsequent rise in circulating plasma alkaline phosphatase levels 4 to 6 hours after start of surgery. The origin of this alkaline phosphatase is attributed to tissue non-specific alkaline phosphatase, most likely liver-type alkaline phosphatase. This endogenous alkaline phosphatase may play a role in the innate immune defence system.

Acknowledgements

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Chapter 6

Anti-inflammatory effects of alkaline phosphatase in patients undergoing elective coronary artery bypass grafting without the use of low dose aprotinin. A pilot study.

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Abstract

Introduction Bovine intestinal alkaline phosphatase administration in patients undergoing elective coronary artery bypass grafting with the use of cardiopulmonary bypass induces endogenous alkaline phosphatase release. However, this was demonstrated in patients where low dose aprotinin was used in the prime fluid. In this study we assessed the effect of bIAP on alkaline phosphatase levels in patients undergoing CABG with the use of CPB without low dose aprotinin.

Methods A total of 9 patients were enrolled and prospectively randomized. Bovine alkaline phosphatase was administered as a bolus of 1000 IU followed by continuous infusion of 5.6 units per kilogram bodyweight per hour for 8 hours (group 1, n=3) or as just a bolus of 1000 IU (group 2, n=3). The other patients received matching placebo (group 3, n=3). The primary endpoint of this small study was to find out if the kinetic profile of endogenous alkaline phosphatase release was mediated by aprotinin. Furthermore, we used two different dosage regimes of bIAP to find out if the endogenous alkaline phosphatase release was bIAP dosage dependent.

Results No significant adverse effects were identified in all 3 groups. In group 1 all patients showed an initial rise of plasma alkaline phosphatase levels due to bolus administration (405,33 ± 108,70 IU/L). A significant increase of plasma alkaline phosphatase at 3-6 hours postoperatively was observed as well (357,33 ± 183,12 IU/L). In group 2 only a significant rise of alkaline phosphatase levels due to bolus administration was found.

Conclusion Intravenous bolus administration followed by continuous infusion for 8 hours induces endogenous alkaline phosphatase release in patients undergoing CABG with the use of CPB, but without low dose aprotinin. Aprotinin is not the eliciting factor for this endogenous alkaline phosphatase release.

1 Introduction

The administration of bovine intestinal alkaline phosphatase (bIAP) in patients undergoing elective coronary artery bypass grafting (CABG) with the use of cardiopulmonary bypass (CPB) was investigated in a prospective, randomized, placebo controlled trial by our group [1]. A total of 63 patients (bIAP n=32, placebo n=31) was randomized to receive either bIAP as an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour, or matching placebo. In that trial all patients received 200 ml, 10000 KIU/mL aprotinin (Bayer Health Care Pharmaceuticals) in the prime fluid of the CPB circuit. Since the use of aprotinin was suspended, based on preliminary results of the BART trial [2] in November 2007, aprotinin is no longer part of standard care in our patients. The use of low dose aprotinin was primarily focused on reduction of blood loss [3;4]. In the literature there is discussion about the potential anti-inflammatory effect of aprotinin [5-8]. In the above mentioned study population we found an overall low inflammatory respone after CABG and only in the bIAP treated group we found in all patients an endogenous alkaline phosphatase release *(Kats et al. Int J Artif Organs, in press)*. We hypothesize that this rise of endogenous alkaline phosphatase in the bIAP treated group might be an effect induced by the use of low dose aprotinin. In this report we describe a small study in patients undergoing elective CABG with the use of CPB, without low dose aprotinin. The primary endpoint of this small study was to find out if the kinetic profile of endogenous alkaline phosphatase release was mediated by aprotinin. Furthermore, we used two different dosage regimes of bIAP to find out if the endogenous alkaline phosphatase release was bIAP dosage dependent.

2 Materials and Methods

In this prospective, randomized, placebo controlled study 9 patients undergoing elective CABG with the use of CPB were randomized to receive either bIAP as an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour at a flow rate of 4 ml per hour for 8 hours (n=3); just an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia(n=3); or matching placebo (n=3). The study was approved by the Institutional Review Board. An informed consent was obtained from each participant.

2.1 Patient selection

After written informed consent was obtained, male or non-pregnant female patients aged ≥ 18 and with a EuroSCORE ≥ 2 and ≤ 6, scheduled to undergo non-emergent coronary artery bypass grafting with the use of cardiopulmonary bypass, were enrolled. Exclusion criteria were redo or emergency operations, baseline alkaline

phosphatase levels > 100 IU/L, evidence of significant hepatic disease or levels of total bilirubine > 34 µmol/L, ALT > 120 U/L or AST > 135 U/L, history or signs of pre-operative infections, immunomodulating medication (i.e. steroids) or patients who were scheduled to receive 'stress dosis' of glucocorticoids, renal failure, creatinin > 177 µmol/L or patients with chronic renal insufficiency requiring dialysis, planned use of leucocyte depletion filtration, preoperative ventilatory support, Body Mass Index > 30, history of idiopathic thrombocytopenia and vegetarians, possibly intolerant of bovine proteins.

2.2 Schedule of assessments

Relevant medical history, concomitant medication and physical examination were obtained at baseline and throughout the study until discharge. Blood samples only for alkaline phosphatase measurement were collected at several time points before, during and after surgery (24 and 0.25 hr before induction of anaesthesia and 0.25 hr, 1.5 hr, 3 hr, 4 hr, 12 hr, 24 hr after induction of surgery, pointed out as visitnumbers 1-8). Perioperative parameters like cross clamp time and perfusion time and clinical parameters like length of ICU stay, duration of ventilation and length of hospital stay were recorded. Adverse events were documented. All clinical laboratory measurements were performed in our hospital.

2.3 Study drug administration

The study drug bovine Intestinal Alkaline Phosphatase (bIAP) or matching placebo, (a sterile solution for infusion containing no bIAP (content 1 ml) in a 2 ml vial in an aqueous buffer containing 20 mM Tris-HCl, 5 mM Magnesium Chloride, 0.1 mM Zinc Chloride, pH 7.3, with 25 % glycerol and human serum albumin as stabilizer) was administered in the first group (Group 1, n=3) as an intravenous bolus of 1000 International Units (IU), just prior to induction of anaesthesia, directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour at a flow rate of 4 ml per hour for 8 hours in order to maintain supranormal levels of alkaline phosphatase in blood. The second group of patients (Group 2, n=3) received bIAP as a single dosis of 1000 International Units (IU), just prior to induction of anaesthesia. The third group (Group 3, n=3) received matching placebo.

2.5 CPB technique

After median sternotomy and preparation of the internal mammary artery, all patients received 3 mg/kg heparin (Leo Pharma, the Netherlands) intravenously. The CPB circuit consisted of a Biomedicus BP80 centrifugal pump (Medtronic, Minneapolis, MN, USA), a membrane oxygenator (Sorin Srl. Avant, Mirandola, Italy or Medtronic Affinity, Minneapolis, MN, USA, or Gish Biomedical, Rancho Santa Margaria, California, USA), a custom made collapsible venous reservoir (Sorin Biomedica, Mirandola, Italy) and a D980 Avant dual chambered hard-shell venous cardiotomy reservoir (Sorin Srl., Mirandola, Italy). Priming fluid consisted of 800 ml

NaCl 0,9%, 500 ml Voluven® (Fresenius Kabi, the Netherlands), 200 ml Mannitol 20% (Baxter Health Care, the Netherlands), 25 ml NaHCO3 8,4% and Heparin 7500 IU. Normothermic cardiopulmonary bypass was applied in all patients. For myocardial protection, depending on the surgeon's preference, either warm blood cardioplegia or St. Thomas cold cristalloid cardioplegia was used. At the end of cardiopulmonary bypass heparin was neutralized with protamine chloride (Valeant Pharmaceuticals, the Netherlands).

3 Results

A total of 9 patients was enrolled in this study. The patients' clinical data are listed in Table 1. No significant safety concerns were identified.
Preoperative levels of alkaline phosphatase were 47,33 ± 9,29 in group 1 (bolus bIAP plus continuous infusion), 65,33 ± 17,50 IU/L in group 2 (bolus bIAP) and 63,0 ± 12,73 IU/L in group 3 (placebo) (p = ns).

	Group 1 (n=3)	Group 2 (n=3)	Group 3 (n=3)
Preoperative data			
Age (y)	73.3 ± 2.3	68.3 ± 6.1	66.7 ± 6.1
Male	1	1	2
BMI (kg/m²)	28.3 ± 6.8	26.1 ± 3.4	30.9 ± 0.7
Euroscore	4 ± 0	2.66 ± 1.15	3.33 ± 1.52
Hx of diabetes (%)	33.3	0	66.6
Operative data			
Number of grafts	3.6	3	3.6
Total duration of surgery (hours)	3.2 ± 0.9	2.9 ± 0.4	2.8 ± 0.4
CPB duration (min)	78.6 ± 25.4	67.3 ± 27.6	72.3 ± 6.7
Cross clamp time (min)	59 ± 9.5	37 ± 26.6	56.7 ± 8.7
Warm blood cardioplegia (%)	33.3	100	100
St. Thomas cold crystalloid cardioplegia (%)	66.6	0	0
Normothermia (%)	100	100	100
Postoperative data			
Intensive care length of stay (hours)	10.5 ± 1.9	28.2 ± 18.9	13.4 ± 7.7
Hospital length of stay (days)	3.6 ± 0.6	4 ± 1.7	4.3 ± 1.5
Postoperative atrial fibrillation (%)	33.3	0	0

Table 1 Baseline characteristics

Group 1: bIAP as an intravenous bolus of 1000 International Units (IU) directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour for 8 hours

Group 2: bIAP as a single intravenous dosis of 1000 International Units (IU)

Group 3: placebo

In group 3 we found in all patients a reduction in alkaline phosphatase levels within 2 hours post induction of surgery (42,0 ± 7,0 IU/L). This reduction in plasma alkaline phosphatase level was followed by normalisation of this level within 24 hours. In group 2 only a significant rise in alkaline phosphatase level was found immediately after bolus administration (510 ± 162,67). No second peak of alkaline phosphatase was found.
In group 1 after the first peak of alkaline phosphatase caused by the bolus (405,33 ± 108,70 IU/L) a second peak of alkaline phosphatase (357,33 ± 183,12 IU/L) was found after 3 to 6 hours (Figure 1).

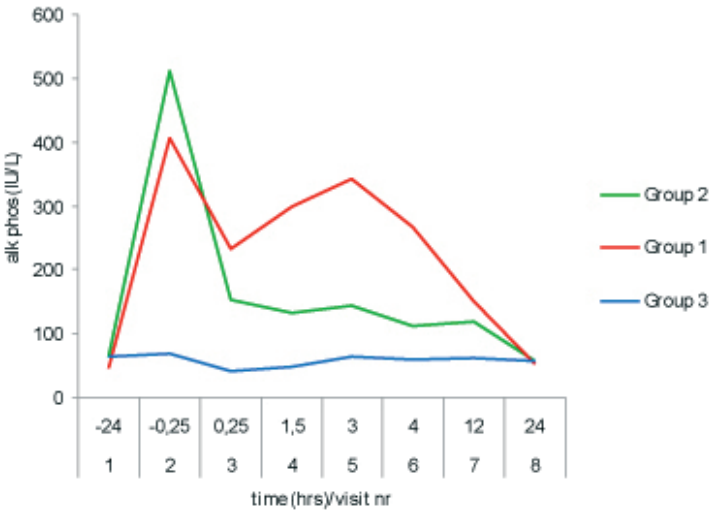

Figure 1 Alkaline phosphatase levels at different time points perioperatively.
Visit numbers correspond to the different time points. Group 1: bIAP as an intravenous bolus of 1000 International Units (IU) directly followed by intravenous continuous infusion of 5.6 units per kilogram bodyweight per hour for 8 hours. Group 2: bIAP as a single intravenous dosis of 1000 International Units (IU). Group 3: placebo

4 Discussion

In this small pilot study we demonstrated that the use of low dose aprotinin in the prime fluid of the CPB circuit is not the eliciting factor for the release of endogenous alkaline phosphatase in patients undergoing elective CABG. Furthermore, this study demonstrates that the endogenous alkaline phosphatase release is only induced by bolus bIAP administration followed by continuous infusion.

The use of aprotinin in cardiac surgery with the use of CPB was primarily focused on reduction of blood loss. Aprotinin has been shown to inhibit plasmin- and kallikrein-mediated fibrinolysis. Furthermore, aprotinin improves haemostasis during and after CPB by preserving the platelet membrane adhesive receptor (glycoprotein Ib) [3;9]. The possible anti-inflammatory effects of aprotinin are induced by inhibition of kallikrein, plasmin, factor XIIq and complement. Aprotinin also decreases the expression of leucocyte integrin CD11b/CD18 and reduces TNFα, IL-8 and IL-6 release in a dosage dependent manner [10].

Because we hypothesize that the endogenous alkaline phosphatase may act like an acute phase protein, where high levels of physiological active alkaline phosphatase have a protective anti-inflammatory effect, we focused in this study on the role of aprotinin in this phenomenon. However, this relation could not be demonstrated. The mechanism of endogenous alkaline phosphatase release thus still remains to be elucidated.

Concerning the different dosage regimens, in this small study it is demonstrated that bIAP bolus administration alone does not induce endogenous alkaline phosphatase release. The intravenously administered bIAP as continuous infusion was calculated to give a doubling of normal plasma alkaline phosphatase levels during the time of perfusion. Apparently a minimum amount of alkaline phosphatase needs to be in the circulation during perfusion to induce this 'de novo synthesis' of alkaline phosphatase.

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Chapter 7

Kinetic profiles of different ways of administration of bovine alkaline phosphatase in piglets

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Abstract

Introduction In patients undergoing elective coronary artery bypass grafting with the use of cardiopulmonary bypass, bolus administration followed by continuous infusion of bovine intestinal alkaline phosphatase (bIAP) induces endogenous alkaline phosphatase release. In this study we tried to induce endogenous alkaline phosphatase release in piglets by different ways of bIAP administration and different dosing regimes.

Methods Piglets received bIAP as a single bolus or as repeated bolus twice a day, with an interval of about 8 hours for 3 consecutive days**.** BIAP was administered intravenously, intramuscularly or subcutaneously in piglets. Alkaline phosphatase levels were determined. L-homo arginine (LHA) was used as a tissue non-specific inhibitor of alkaline phosphatase to demonstrate the presence of endogenous alkaline phosphatase.

Results After intravenous bIAP administration alkaline phosphatase levels drop to base levels quickly, in consistence with the short half life time of bIAP. After single subcutaneous bolus administration alkaline phosphatase levels remain elevated for a few hours. In repeated bolus administration both subcutaneously and intramuscularly, after each bolus a slight elevation of base levels of alkaline phosphatase exists. In all experiments the detected plasma alkaline phosphatase was LHA resistant and thus most likely represents the administered alkaline phosphatase.

Conclusion Endogenous alkaline phosphatase can not be induced by repeated intravenous bIAP administration, nor by intramuscular single bolus or repeated intramuscular bolus administration, nor by subcutaneous single bolus or repeated intramuscular bolus administration.

1 Introduction

Since the first publication of possible beneficial effects of alkaline phosphatase in dephosphorylation of lipopolysaccharide by Poelstra in 1979 [1;2], alkaline phosphatase administration has gained much interest for research in inflammatory diseases. In several animal models for inflammation beneficial effects of administration of alkaline phosphatase are reported [3-9]. Moreover, promising results of alkaline administration are achieved in humans in a septic shock [10] and in patients with moderate to severe ulcerative enterocolitis[11]. In 2009 we reported on the possible beneficial effect of bovine intestinal alkaline phosphatase in patients undergoing elective coronary artery bypass grafting (CABG) [12]. Alkaline phosphatase has once been called 'an emperor with new clothes' [13] since the real mechanism of action in septic renal injury has not yet been elucidated [10]. In the different reports mentioned above different ways of alkaline phosphatase administration are used. In studies performed in humans either intravenous administration of alkaline phosphatase, or intraduodenal administration was used [10;12;14;15]. In animal studies either intraperitoneal or intravenous administration was used [3;5-7;9]. When alkaline phosphatase was administered intravenously in humans different dosing regimes were used. Alkaline phosphatase was administered as a bolus, as continuous infusion, either with or without preceding bolus infusion or as continuous infusion only [10;12;15]. In her thesis on safety and efficacy of bovine calf intestinal alkaline phosphatase, Beumer reported on a study among different animals resulting in animal species-dependent antibody formation and animal species-dependent toxic effects [16]. Treatment of piglets with bIAP for 28 days by repeated bolus injection of 4000 IU bIAP each day did not result in any adverse effects [3]. In mice, however, repeated intravenous bolus administration of 750 IU/kg or 7500 IU/kg lead to death at day nine of the study. However, when mice were administered bIAP in drinking water for 4 weeks prior to repeated bolus administration, death did not occur.
In patients undergoing elective coronary artery bypass grafting (CABG) and receiving alkaline phosphatase as a bolus followed by continuous infusion an endogenous alkaline phosphatase release was found as detected by iso-enzyme analysis [17] (Figure 1) *(Kats et al. Int J Artif Organs, in press)*.This endogenous alkaline phosphatase release is an intriguing finding of which the mechanism and origin have not yet been elucidated. In this animal study in piglets different ways of administration of bIAP, as well as different dosing regimens and the associated pharmacokinetics are described. The primary endpoint of this study was to induce endogenous alkaline phosphatase release in the same way as we found it in CABG patients[12].

2 Methods

The study was approved by the animal ethics committee of the Utrecht University. The experiments were performed according to the Dutch legislation on protection of animals.

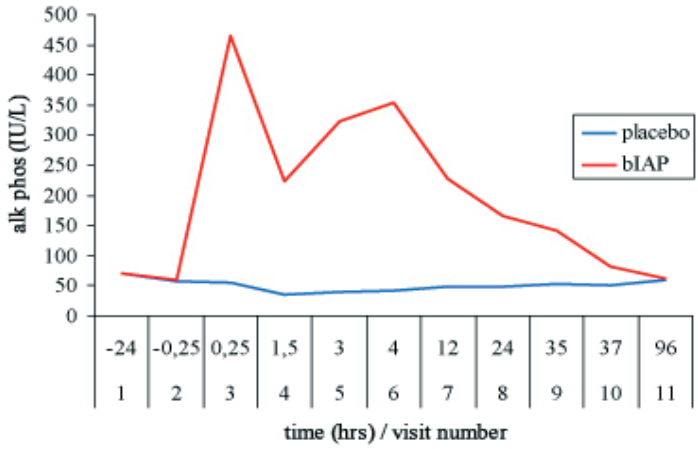

Figure 1 Alkaline phosphatase levels at different time points perioperatively in patients undergoing elective CABG. Visit numbers correspond to the different time points perioperatively.

Two Female Dutch Landrace x Yorkshire piglets were housed under conventional conditions at the animal housing facility of the university of Utrecht with a 12 h light/dark regime and fed a standard ration[3].
To facilitate blood sampling and intravenous bIAP administration the jugular vein of each piglet was surgically catherized. After surgery piglets were allowed to recuperate one week in which they received antibiotics (Praxavet and Finedyne).
BIAP was administered in three different manners. In the first experiments we administerd bIAP intravenously through the jugular vein catheter.
After that we performed experiments with intramuscularly administered bIAP.
Finally, we administered bIAP subcutaneously. Piglets were sedated with propofol (Astra Zeneca®, Zoetermeer, The Netherlands) for about two minutes during subcutaneous and intramuscular bIAP administration.
After each experiment piglets were allowed to recuperate for 10 to 14 days, to be sure that all administered bIAP had disappeared from the piglets body. Thus, in practice we performed one experiment every two weeks.
Blood sampling was carried out under aseptic conditions. Blood samples were taken at different time intervals after bIAP administration from the vena jugularis catheter, which was rinsed with heparin (10 IE/ml 0.9% NaCl) to prevent blood clotting.
In addition to blood sampling piglets were monitored for clinical parameters and well being during all experiments.

2.1 BIAP administration

Because the induction of endogenous alkaline phosphatase was only seen in patients who received bIAP as a bolus followed by continuous infusion, the most obvious

experiment in piglets was to give bIAP as an intravenous bolus of 1000 IU followed by continuous infusion of 1000 IU bIAP for 4 hours. Next to that we administered repeated intravenous bolus of 1000 IU for 3 days at time points 0,8,24,32,48 and 56 hours.
Previous experiments by Beumer et al. showed that repeated doses of intravenously administered bIAP (4000 IU per day for 28 days) did not have statistical significant influence on haematological parameters, clinical chemistry and electrolyte balance in piglets[3].
In order to obtain intramuscular bIAP pharmacokinetics, piglets received bIAP as a bolus of 1000 IU intramuscularly. Next to that we administered repeated intramuscular bolus of 1000 IU bIAP for 3 days at time points 0,8,24,32,48 and 56 hours.
In the same way we did experiments with subcutaneous bIAP administration. Thus, bIAP was administered subcutaneously as a single bolus of 10000 IU, or as repeated bolus of 10000 IU for 3 days at time points 0,8,24,32,48 and 56 hours.
A schedule of assessments for the different experiments is depicted in Table 1.

Days	1		2		3	
Hours	0	8	24	32	48	56
Intravenous						
1000 IU + cont infusion 1000 IU	x »					
1000 IU (repeated)	x	x	x	x	x	x
Intramuscular						
8000 IU	x					
1000 IU (repeated)	x	x	x	x	x	x
Subcutaneous						
10000 IU	x					
10000 IU (repeated)	x	x	x	x	x	x

Table 1 Schedule of assessments for bIAP administration in the different experiments in piglets

2.2 Alkaline phosphatase activity

Alkaline phosphatase activity was determined using a PNPP (p-nitrophenol phosphate) kinetic assay [18]. Samples were defrosted and warmed gradually to 21 degrees Celsius. Two hundred µliter of a serum sample was mixed with 1 ml of PNPP-substrate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and $MgCl_2$ (final concentration 2 mM) in a Tris-glycin buffer at pH 9.6. Samples were measured kinetically at 405nm on Biorad Smartspec photo spectrometer for 60 seconds with intervals of 20 seconds.

2.3 L-homo arginine (LHA) inhibition

To demonstrate the presence of endogenous alkaline phosphatase L-homo arginine (LHA) (Sigma-AldrichChemie BV, Zwijndrecht, The Netherlands) was used as a tissue non-specific alkaline phosphatase (TNSALP) inhibitor. LHA is supposed to have minimal influence on bIAP, but inhibits tissue non-specific alkaline phosphatase activity [19]. Pig plasma was diluted to a concentration of 0.08 U/L and 100 µL was added to a cuvette. Five mM LHA (final concentration) was added and this was gently mixed and incubated at room temperature for 5 minutes. Next a PNPP Kinetic assay was carried out similarly as described above [18], but now measurements lasted 180 seconds with intervals of 20 seconds.
LHA inhibition was performed in repeated bolus experiments in intravenous, intramuscular and subcutaneous bIAP administration.

3 Results

No adverse effects of either way of bIAP administration were determined.
Mean alkaline phosphatase plasma level at onset of the study was 116 IU/L

3.1 Intravenous bIAP administration

Bolus infusion of 1000 IU bIAP followed by continuous infusion of 1000 IU bIAP for 4 hours resulted in a fast peak caused by bolus infusion followed by fast clearance from the circulation. After this fast elimination plasma alkaline phosphatase levels remain slightly elevated for about the time of continuous bolus bIAP infusion (4 hours/240 minutes). However a second peak of alkaline phosphatase as demonstrated in CABG patients was not seen (Figure 2)

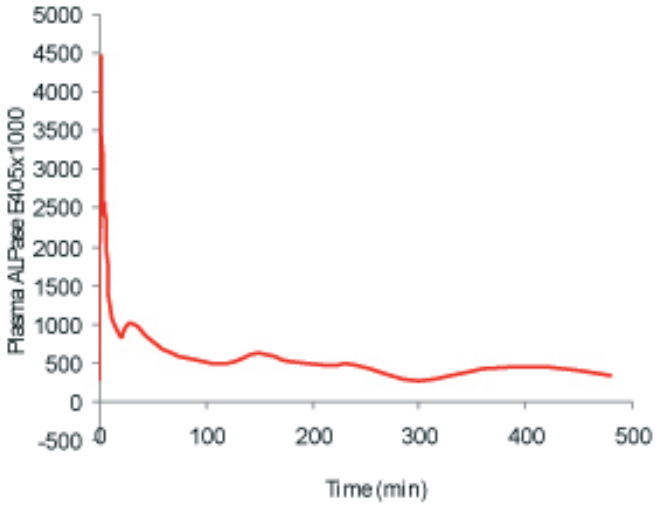

Figure 2 Bolus administration followed by continuous infusion of 1000 IU bIAP for 4 hours

In repeated intravenous bIAP administration (1000 IU) for 3 days at time points 0,8,24,32,48 and 56 hours it is noted that after each intravenous bolus total plasma alkaline phosphatase level falls back to base levels within minutes (Figure 3, continuous line). LHA inhibition studies performed in plasma after repeated intravenous bolus administration are shown in Figure 3. It is demonstrated that all detected alkaline phosphatase represents the administered bIAP. No endogenous alkaline phosphatase release was induced after repeated intravenous bIAP administration.

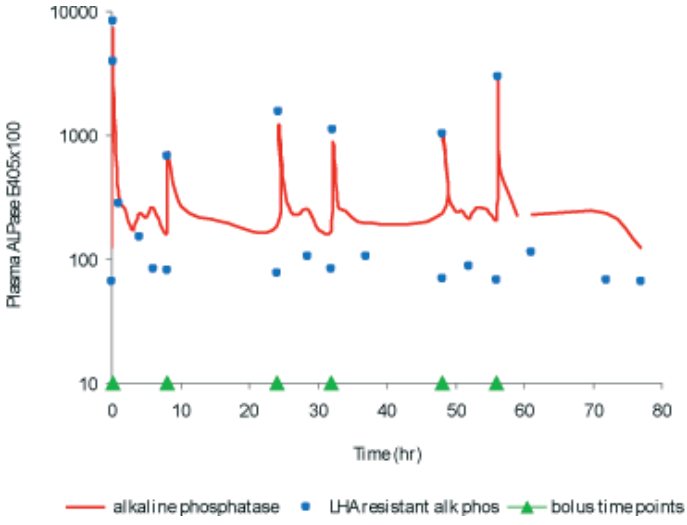

Figure 3 Repeated intravenous bIAP (1000 IU) administration for 3 days at time points 0,8,24,32,48 and 56 hours and LHA inhibition

3.2 Intramuscular bIAP administration

Piglets were sedated with propofol (Astra Zeneca®, Zoetermeer, The Netherlands) for about two minutes during intramuscular bIAP administration in the groin cavity or armpit.

Figure 4 shows the pharmacokinetics of intramuscular bolus injection of 8000 IU bIAP. Systemic availability of single dose intramuscular bIAP administration is within minutes. After the first peak a drop in alkaline phosphatase level till a two fold base level persists for about 6 hours.

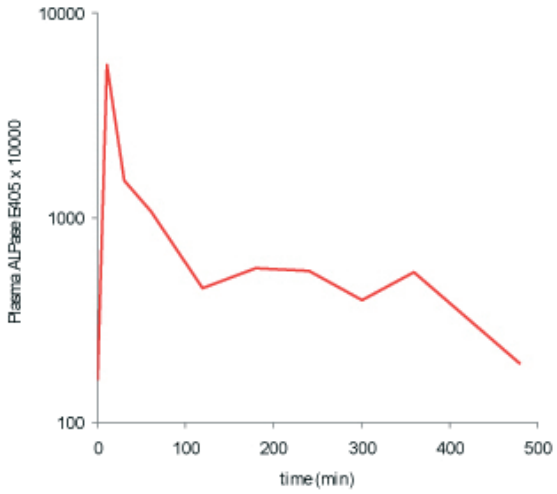

Figure 4 BIAP intramuscular bolus (8000 IU) pharmacokinetics

Repeated intramuscular bolus bIAP injection of 1000 IU at time points 0,8,24,32,48 and 56 hours is depicted in Figure 5 (continuous line). After each intramuscular injection the alkaline phosphatase level rises quickly, followed by a drop in alkaline phosphatase level to about two fold base level. This 'fall back level' is slightly more elevated after each intramuscular injection. LHA inhibition of repeated intramuscular bIAP administration is demonstrated in Figure 5. The induction of endogenous alkaline phosphatase could not be confirmed.

3.3 Subcutaneous bIAP administration

Figure 6 shows the increase in plasma alkaline phosphatase levels in time relative to the plasma level at onset of the study after single subcutaneous bolus administration of 10000 IU bIAP. Single dose subcutaneous administration results in peak plasma levels after 3 hours. The alkaline phosphatase levels are increased for a prolonged period up to 8 to 9 hours after administration and slowly return to baseline level after about 15 hours.

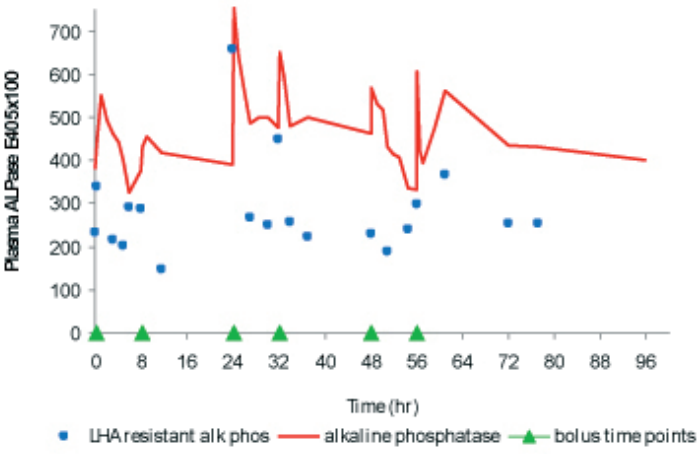

Figure 5 Repeated intramuscular bIAP administration (1000 IU) at time points 0,8,24,32,48 and 56 hours and LHA inhibition

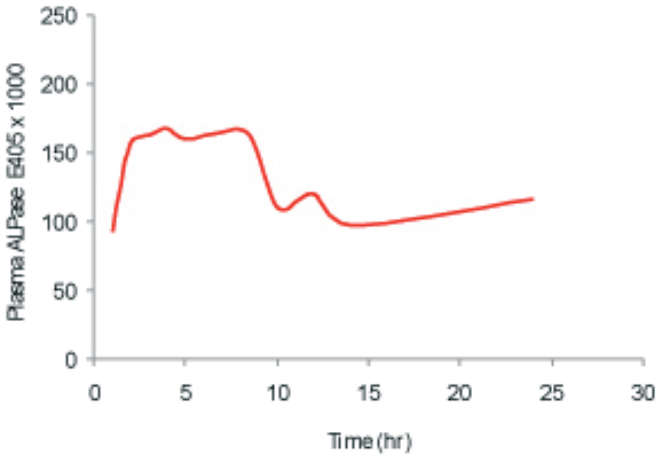

Figure 6 Single subcutaneous bIAP bolus (10000) IU

Repeated subcutaneous administration of 10000 IU bIAP twice a day, with an interval of about 8 hours for 3 consecutive days leads to a kinetic profile as shown in Figure 7 (continuous line). The amount of alkaline phosphatase does not return to baseline levels between different subcutaneous administration. In fact, after 4 subcutaneous administrations it seems that the baseline alkaline phosphatase

level is increased. After repeated subcutaneous administration of bIAP alkaline phosphatase levels return to baseline levels after more than 30 hours. LHA inhibition of repeated subcutaneous bIAP administration is shown in Figure 7. The induction of endogenous alkaline phosphatase could not be confirmed.

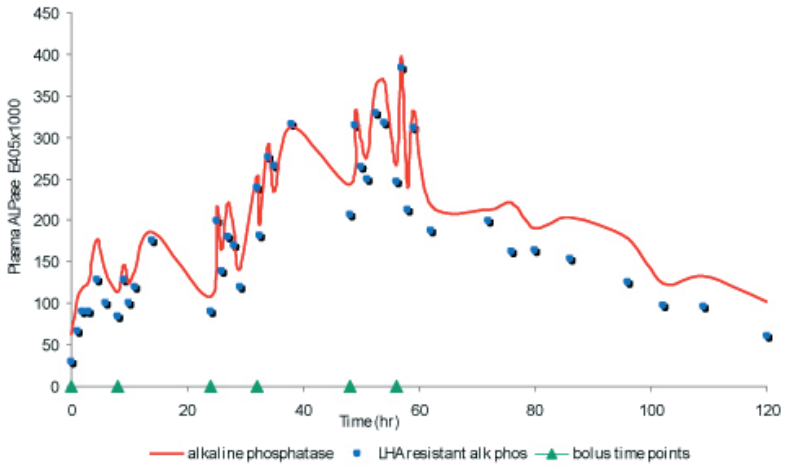

Figure 7 Repeated subcutaneous bIAP administration (10000 IU) at time points 0,8,24,32,48 and 56 hours and LHA inhibition

4 Discussion

In this animal study among piglets different ways of bIAP administration and corresponding pharmacokinetics are demonstrated. In a previous report we demonstrated the induction of endogenous alkaline phosphatase after bolus bIAP administration followed by continuous infusion for at least 8 hours in patients undergoing elective CABG (ref Int J Artif Organs). The origin of this endogenous alkaline phosphatase is under investigation and so far not exactly known.
The primary endpoint of this animal study was to induce endogenous alkaline phosphatase in different ways of bIAP administration and dosage.
As demonstrated by Beumer et al.plasma elimination of intravenously administered

bIAP as a bolus in piglets consists of two phases. This biphasic clearance knows a fast initial and a slower second elimination of bIAP from the circulation [3].
Since alkaline phosphatase is also produced endogenously it is difficult to evaluate pharmacokinetics of exogenously administered alkaline phosphatase. To distinguish between administered and endogenous alkaline phosphatase we used LHA inhibition in the different models.
The most obvious way in the attempt of inducing endogenous alkaline phosphatase was intravenous bolus administration followed by continuous infusion. However, a second peak of alkaline phosphatase could not be demonstrated in piglets.
Repeated intravenous bolus administration showed a fast drop till base levels after each bolus followed by a new peak level by renewed bolus administration. It confirms the short half-life time of bIAP of approximately 10 minutes.
In the experiments with intramuscular bIAP, bolus administration shows a prolonged high level of alkaline phosphatase up till twice the base level for a period of about 6 hours. In the case of repeated intramuscular bIAP administration the biphasic plasma elimination described by Beumer et al. is confirmed [3]. Thus, after each bolus plasma alkaline phosphatase level remains higher than baseline level and alkaline phosphatase is not completely cleared before the next bolus is administered. This leads to a slowly rising baseline level.
It is hypothesized by Beumer et al. that bIAP binds to the endothelial wall of the blood vessels causing a slow release of bIAP in the second phase of bIAP clearance [3]. In intramuscular administration depot formation might play a role in the slow release of bIAP.
It is demonstrated that subcutaneous high dose (10000 IU) bIAP administration leads to prolonged high levels of bIAP even up to 8 hours post administration. The prolonged high level of subcutaneously administered bIAP might be the result of depot formation of alkaline phosphatase in subcutaneous tissue. However, we were not able to demonstrate endogenous alkaline phosphatase release in either one of the performed experiments. In all experiments the detected plasma alkaline phosphatase was LHA resistant and thus most likely represents the administered alkaline phosphatase.
Despite different dosing and administration regimens of bIAP in piglets, the release of endogenous alkaline phosphatase in patients undergoing elective CABG and treated with bIAP bolus followed by continuous infusion remains unclarified and a subject for future investigation.

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Chapter 8

Summary
General discussion and future perspectives

Summary

Alkaline phosphatase is an endogenous ecto-enzyme, widely expressed in many organs that are exposed directly or indirectly to the external environment, like the gastrointestinal tract and the lungs. There are two types of alkaline phophatases. Tissue-non specific alkaline phosphatases (TNSALP) include bone-, liver- and kidney isotypes. Tissue specific alkaline phosphatases include human placental alkaline phosphatase (HPLAP)-, germ cell (GCAP)-, and intestinal (IAP) isotypes. Different isotypes serve different functions at different sites in the body [1]. A physiological role for alkaline phosphatase was proposed in 1997 by Poelstra et al. [2;3] and was confirmed by Bentala et al. in 2002 [4]. Alkaline phosphatase dephosphorylates and thereby detoxifies not only endotoxins (lipopolysaccharides) but also extracellular nucleotides [4;5]. The phosphorylated lipid-A moiety of endotoxin is a substrate for alkaline phosphatase, which enzymatically dephosphorylates the toxic lipid-A part into monophosphoryl lipid-A, a non-inflammatory metabolite, and inorganic phosphate [3;4]. Furthermore, alkaline phosphatase converts nucleotides into non-inflammatory nucleosines [6]. Both endotoxins and nucleotides are potent inflammatory triggers and are sensed as 'stranger'or 'danger' signals to the innate immune system, and subsequently local and systemic inflammatory responses (SIRS) may result from the exposure to these pro-inflammatory signals [7].

In various animal studies promising therapeutic effects in reducing the inflammatory response with the use of intravenous alkaline phosphatase are shown [8-11]. In a clinical study in severe sepsis patients continuous infusion of calf-intestinal alkaline phosphatase significantly improved renal function [12]. In patients with severe ulcerative colitis exogenous alkaline phosphatase improved short-term disease activity scores[13]. More recently a protective role of intestinal alkaline phosphatase has been demonstrated in necrotizing enterocolitis in an experimental rat model [14].

After myocardial infarction complicated by cardiogenic shock the inflammatory response is the result of an ischemic insult [15]. Elevated procalcitonin levels, correlated to IL-6 levels, have been described after acute myocardial infarction. It is well known that bacterial toxins are by far the most potent trigger for elevated procalcitonin levels [16]. In cardiac surgery with the use of cardiopulmonary bypass (CPB) hypoperfusion of the gut may result in a loss of barrier function and as a consequence bacterial endotoxins, normally confined to the lumen of the intestine by a barrier of endovascular cells, may enter the systemic circulation [17-19]. Laboratory and clinical data have implicated endotoxin as an important factor in the inflammatory reaction post CPB.

Based on the promising results of alkaline phosphatase in the attenuation of the inflammatory response in several studies we focused on the effect of bovine Intestinal Alkaline Phosphatase (bIAP) on the inflammatory response in the field of cardiology and cardiac surgery.

Bovine calf intestinal mucosa represents a rich source of intestinal alkaline phosphatase (bIAP). The study drug bIAP used in this thesis was manufactured by

Biozyme ltd (Bleanavon, Wales, UK) and Alloksys Life Sciences B.V. (Bunnik, The Netherlands).

The whole body inflammatory response is known to be both material dependent, caused by exposure of blood to nonphysiologic surfaces and conditions, and material independent, caused by the surgical trauma itself, ischemia-reperfusion, changes in body temperature and the release of endotoxin. In **chapter 2** of this thesis the pathofysiology and possible therapeutic strategies to attenuate the whole body inflammatory response are reviewed, focusing on endotoxin release. The proposed mechanism of endotoxin release during cardiac surgery with the use of CPB is discussed, followed by both material dependent and material independent measures taken throughout the years. Thus, humoral factors i.e. numerous receptors of innate immunity (Lipopolysaccharide Binding Protein (LBP), CD-14 and Toll Like Receptor-4 (TLR-4)) and anti-endotoxin antibodies (IgG- and IgM EndoCab) are discussed as well as pharmaceutical strategies, endotoxin adsorbers in the CPB system and CPB related factors as hypothermia and off pump surgery. Although many strategies to attenuate the endotoxin release have been investigated, none of them were able to demonstrate any clinical signicant improvement.
In **chapter 3** we investigated the anti-inflammatory effect of bIAP in myocardial infarction. The left anterior descending (LAD) ligation model was used to induce acute myocardial infarction (AMI) in mice. Bovine intestinal alkaline phosphatase was given intravenously as a prophylaxis. Pro-inflammatory cytokines IL-6 and IL-1β were significantly reduced in the acute phase after myocardial infarction in mice receiving bIAP as compared to the non-bIAP treated mice. After acute myocardial infarction in mammals, mast cells are activated to release chymases. Activation of mast cells in mice was measured by the release of the mouse mast cell protease-1 (mMCP-1) chymase. Mouse mast cell protease-1 chymase production was decreased in bIAP treated mice as compared to non-bIAP treated mice. In conclusion a single intravenous dose of bIAP reduced the production of the chymase mMCP-1 by mast cells and diminished the systemic pro-inflammatory cytokine response in the acute phase after AMI and might represent a novel therapeutic drug in attenuating the pro-inflammatory response after AMI. During cardiac surgery with the use of cardiopulmonary bypass, mesenteric hypoperfusion occurs which results in a loss of barrier function and as a consequence bacterial endotoxins may enter the systemic circulation. In **chapter 4** the APPIRED study (Alkaline Phosphatase in Prevention of Ischemia Reperfusion Damage) is discussed. In a double blind, randomized, placebo-controlled study, a total of 63 patients undergoing coronary artery bypass grafting (CABG) with low till intermediate EuroSCORE were enrolled. Bovine intestinal alkaline phosphatase or placebo was administered as an intravenous bolus followed by continuous infusion for 36 hours. The primary endpoint was reduction of post-surgical inflammation. Furthermore, clinical parameters were assessed. The overall inflammatory response to CABG with the use of CPB was low in both placebo and bIAP treated patient group. Five patients

in the placebo group displayed a significant TNFα response followed by an increase in plasma levels of IL-6 and IL-8. Such a TNFα response was not observed in the bIAP group, confirming the anti-inflammatory activity of bIAP. The anti-inflammatory cytokines IL-10, IL-2 and IL-4 were not affected. Other variables related to systemic inflammation showed no statistically significant differences. There was no significant difference in clinical outcome between the two groups. In **chapter 5** a substudy of the APPIRED study is discussed in which we focused on alkaline phosphatase release and hypothesized that administration of bIAP would increase endogenous alkaline phosphatase release in patients undergoing CABG with the use of CPB. The intravenous administration of bIAP as a bolus followed by continuous infusion to patients undergoing elective CABG with CPB leads to an initial rise of plasma alkaline phosphatase levels due to bolus administration followed by a significant increase of plasma alkaline phosphatase at about 4-6 hours post start of surgery. This second peak of alkaline phosphatase has an endogenous origin.
We were able to inhibit the enzymatic activity of alkaline phosphatase in this second peak with L-homo arginine (LHA). Therefore, this second peak is caused by the generation of Tissue Non Specific Alkaline Phosphatase (TNSALP-type alkaline phosphatase).Through isoenzyme analysis it was excluded that this post surgical rise of plasma alkaline phosphatase could be attributed to a rise of bone type alkaline phosphatase. Hence the most likely source is liver type alkaline phosphatase. This endogenous alkaline phosphatase, which was noted only in patients treated with a bIAP bolus followed by continuous infusion, may play a role in the innate immune defence system and is a unique finding that to our knowledge has not been described before. In the APPIRED study low dose aprotinin was added to the prime fluid in all patients. Since the use of aprotinin has been hampered by concerns about safety and costs, it is no longer standard care in our patients. To exclude that aprotinin as possible anti-inflammatory agent was the eliciting factor in endogenous alkaline phosphatase release we performed a small study in patients undergoing CABG with the use of CPB, but without the use of aprotinin. This small study is described in **chapter 6**. Nine patients were enrolled to receive either bIAP as a bolus, or bIAP as a bolus followed by continuous infusion, or matching placebo. In this study it is demonstrated that the release of endogenous alkaline phosphatase is not induced by aprotinin, and that it is only induced by bolus bIAP followed by continuous infusion. The intriguing finding that only bolus plus continuous infusion of bIAP induces endogenous alkaline phosphatase release urged us to search for different dosage regimes and different ways of bIAP administration in piglets. The results of these experiments are described in **chapter 7**. In piglets bIAP was administered intravenously, intramuscularly or subcutaneously either as a single bolus or as repated bolus twice a day, with an interval of about 8 hours for 3 consecutive days. After intravenous bIAP administration alkaline phosphatase levels drop to base levels quickly, in consistence with the short half life time of bIAP. After single subcutaneous bolus administration alkaline phosphatase levels remain elevated for a few hours. In

repeated bolus administration both subcutaneous and intramuscular after each bolus a slight elevation of base levels of alkaline phosphatase occurs. In all experiments the detected plasma alkaline phosphatase was LHA resistant and thus most likely represents the administered alkaline phosphatase. No endogenous alkaline phosphatase release could be demonstrated in piglet experiments.

General discussion and future perspectives.

Since the first publication by Poelstra et al. of dephosphorylation of endotoxin by alkaline phosphatase in 1997 [2;3], extensive research has been done to elucidate the mechanism of dephosphorylation [20]. Dephosphorylation by alkaline phosphatase is a key mechanism in host defence against inflammation, both due to exogenous toxins like endotoxin and to endogenous extracellular ATP and other nucleotides [5;21]. Promising therapeutic effects of intravenously administered alkaline phosphatase have recently been published [8;12-14;22].

In this thesis a possible role for alkaline phosphatase in the attenuation of the inflammatory response after acute myocardial infarction (AMI) is proposed. Four hours after AMI, a significant reduction in the concentrations of the two most prominent pro-inflammatory cytokines present in serum in the acute phase after AMI, IL-6 and IL-1β, was observed when compared to non-bIAP treated controls. A reduction in pro-inflammatory cytokine production indicates a diminished systemic innate immune response, which may decrease myocardial dysfunction and reduce the development of cardiogenic shock after AMI [23]. Moreover, the noticed effect of bIAP on mMCP-1 production might result in a reduction of angiotensin II formation and consequently a decrease in arrhythmias, which may improve cardiac function and reduce cardiogenic shock complications. Although we could not demonstrate the direct effect of bIAP on LPS in this study, the specific activity of human placental alkaline phosphatase HPLAP and bIAP against an LPS insult has been undoubtedly demonstrated in vivo [4;14;24;25]. The role of endotoxin in the whole body inflammatory response as a consequence of cardiac surgery with the use of CPB has been well established [18;19;26;27]. In the APPIRED study in patients undergoing elective coronary artery bypass grafting with the use of CPB, the overall inflammatory response appeared to be low in both the bIAP and the placebo group. In several clinical trials higher levels of pro-inflammatory cytokines are reported [28;29]. Endotoxin release followed by an increase in pro-inflammatory cytokines is related to CPB time and aortic cross clamp time [26;30]. In our study mean CPB time and aortic cross clamp time were possibly too short to lead to a significant increase in pro-inflammatory parameters. However, in the placebo group, 5 patients showed a significant TNFα response with a subsequent rise in IL-6 and IL-8, which was not seen in any of the bIAP-treated patients. This strongly suggests that there might be a role for bIAP in CABG patients in reducing the post-surgical inflammatory response. It would be of interest to see if patients undergoing cardiac surgery with the use of CPB with expected longer cross clamp time and perfusion time show more inflammation and

thus might benefit more from treatment with bIAP. In fact, the APPIRED II study was started in January 2010. In this randomized, double blind, placebo controlled study 50 patients undergoing elective aortic valve replacement combined with CABG will be enrolled. The study has been approved by the the Institutional Review Board in December 2009. Patients receive either bIAP as a bolus followed by continous infusion or matching placebo. Primary endpoint is reduction in inflammation. Next to that the effect of bIAP on clinical outcome will be studied.

The most intriguing finding in this thesis is the release of endogenous alkaline phosphatase, till now only seen in patients undergoing elective CABG with the use of CPB and receiving bIAP as a bolus followed by continuous infusion for at least 8 hours. In different ways of administration of bIAP in piglets and with different dosing regimes we were not able to induce endogenous alkaline phosphatase release. We tried to determine the origin of this endogenous released alkaline phosphatase in the bIAP treated group and also to explain the fact that only a bolus bIAP followed by continuous infusion leads to this endogenous alkaline phosphatase release in patients undergoing elective CABG with th use of CPB. By inhibition assays we could establish that the most likely source of this endogenous alkaline phosphatase is liver type alkaline phosphatase, one of the tissue non-specific alkaline phosphatase group. The fact that for hemodilution corrected bilirubin levels were slightly elevated strengthened our feeling about this source.

Tuin et al. demonstrated in vitro that LPS exposure to liver slices in situ results in increased mRNA for alkaline phosphatase expression with kinetics that are compatible with de novo synthesis [31]. We hypothesize that alkaline phosphatase prophylaxis improves the defence mechanism against a new inflammatory insult by triggering the release of sustainable alkaline phosphatase in the circulation possibly as a consequence of de-novo synthesis.

Thus, alkaline phosphatase may act like an acute phase protein, where high levels of physiological active alkaline phosphatase have a protective anti-inflammatory effect. The pre-operative plasma levels may predict clinical outcome in acute inflammation in a manner similar to that reported for high plasma anti-endotoxin antibody levels [28;32;33]. Patients might be protected by pre-treatment with physiologically active alkaline phosphatase which will elevate their endogenous physiological levels. Although we demonstrated that bovine intestinal alkaline phophatase can be administered safely in humans and is well tolerated, there might be some concerns about its bovine origin. In 2006 Bodrogi et al. [34] reported on the production of high level enzymatically active human tissue non-specific alkaline phosphatase in the milk of transgenic rabbits. Recently this recombinant human tissue non-specific alkaline phosphatase has been proven effective in lipopolysaccharide-induced systemic inflammatory response syndrome in an in-vivo mouse model of sepsis (data to be published). Thus, recombinant human tissue non-specific alkaline phosphatase might be the ideal therapeutic alkaline phosphatase in attenuating the inflammatory response from any cause.

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Chapter 9

Nederlandse samenvatting

Alkalische fosfatase is een enzym dat in vele organen tot expressie komt. Er zijn twee soorten alkalische fosfatase beschreven, weefsel specifiek alkalische fosfatase en niet-weefsel specifiek alkalische fosfatase. Tot de weefsel specifieke alkalische fosfatases behoren placentair-, geslachtscel- en intestinaal alkalische fosfatase. Tot de niet-weefsel specifieke alkalische fosfatases horen bot-, lever-, en nier alkalische fosfatase. De verschillende isotypes hebben verschillende functies op verschillende plaatsen in het lichaam. In 1997 werd aangetoond dat alkalische fosfatase een rol speelt bij de defosforylering en dus detoxificering van endotoxines. Alkalische fosfatase is in staat om één fosfaatgroep van het gefosforyleerde lipid A gedeelte van endotoxines "af te knippen" en daardoor om te zetten in monofosforyl lipid A, dat geen ontstekingsreactie opwekt, en inorganisch fosfaat. Daarnaast is er een rol voor alkalische fosfatase beschreven in de omzetting van nucleotides in niet toxische nucleosines. Zowel endotoxines als nucleotides vormen een bedreiging voor het lichaam, aangezien zij een inflammatoire reactie induceren op lokaal en systemisch niveau.
In diverse studies zijn veelbelovende resultaten van alkalische fosfatase toediening in het reduceren van de inflammatoire reactie beschreven. In een klinische studie bij patiënten met een ernstige sepsis verbeterde een continu infuus met alkalische fosfatase significant de nierfunctie. Zeer recent is een positief effect van alkalische fosfatase toediening in een zogenaamd rat model van necrotiserende enterocolitis beschreven.
Na een acuut myocard infarct treedt een inflammatoire reactie op die waarschijnlijk het gevolg is van verminderde perfusie van de darmen door verminderde cardiac output als gevolg van verminderde hartfunctie. Dit resulteert waarschijnlijk in translocatie van endotoxines in de systemische circulatie.
De rol van endotoxine in de hartchirurgie is uitgebreid bestudeerd in de jaren 90. Het gebruik van de hartlong machine veroorzaakt een inflammatoire reactie die deels materiaal afhankelijk en deels niet-materiaal afhankelijk is. De materiaal afhankelijk geïnduceerde inflammatoire reactie wordt met name veroorzaakt door het contact van bloed met het 'vreemd lichaamsoppervlak' van de hartlong machine. De niet-materiaal afhankelijk geïnduceerde inflammatoire respons wordt bepaald door het chirurgisch trauma, ischemie-reperfusie schade en het vrijkomen van endotoxine. Intensief onderzoek naar de materiaal afhankelijke factoren heeft geleid tot een aanzienlijke verbetering van de tegenwoordig gebruikte hartlong machines. Met heparine gecoate slangen, het gebruik van mini circuits en cell saving is de inflammatoire reactie na gebruik van de hartlong machine fors verminderd. Ook op het gebied van de niet-materiaal afhankelijke factoren is uitgebreid onderzoek verricht, welke zich voor een groot deel gericht heeft op het vrijkomen van endotoxine.

Gebaseerd op de veelbelovende resultaten van toediening van alkalische fosfatase in het verminderen van de inflammatoire reactie werd in dit proefschrift de toediening

van alkalische fosfatase onderzocht op het gebied van de cardiologie en de cardiothoracale chirurgie. Het onderzochte bovine intestinaal alkalische fosfatase (bIAP) werd geproduceerd door Biozyme ltd (Bleanavon, Wales,Engeland) en Alloksys Life Sciences B.V. (Bunnik, Nederland).

Hoofdstuk 1 bevat de algemene introductie. In **hoofdstuk 2** wordt een overzicht gegeven van de literatuur over het bestrijden van de inflammatoire reactie als het gevolg van het gebruik van de hartlong machine. In dit hoofdstuk ligt de nadruk op het bestrijden en voorkomen van de effecten van het vrijkomen van endotoxine. Getriggerd door zowel materiaal afhankelijke en niet-materiaal afhankelijke factoren. Zo worden humorale factoren beschreven zoals receptoren (lipopolysaccharide binding protein, CD-14 an Toll Like Receptor-4) en anti endotoxine antilichamen (IgG- en IgM EndoCab). Daarnaast worden pharmaceutische strategieën, endotoxine adsorberende filters en hartlong machine gerelateerde factoren zoals hypothermie en zogenaamde off pump chirurgie besproken. Ondanks het feit dat er veel onderzoek is gedaan naar het bestrijden van de inflammatoire effecten als gevolg van het vrijkomen van endotoxine, heeft geen van de beschreven onderzoeken geleid tot een verbetering van morbiditeit en mortaliteit na hartchirurgie met gebruik van de hartlong machine.

In **hoofdstuk 3** hebben we het anti-inflammatoire effect onderzocht van bovine intestinaal alkalische fosfatase (bIAP) na een acuut myocard infarct. Bij muizen werd een acuut myocard infarct geïnduceerd door de left anterior descending (LAD) kransslagader te onderbinden. BIAP werd intraveneus toegediend als prophylaxe. De pro-inflammatoire cytokines IL-6 en IL-1β waren significant verminderd in de muizen die met bIAP waren behandeld in vergelijking met de muizen die niet met bIAP waren behandeld. Na een acuut myocard infarct worden mestcellen geactiveerd om chymase af te geven. In het muis model werd de activatie van mestcellen gemeten aan het vrijkomen van muis mestcel protease-1 (mMCP-1) chymase. Muis mestcel protease-1 (mMCP-1) chymase productie was verminderd in muizen met bIAP behandeld in vergelijking met muizen die niet met bIAP waren behandeld. De conclusie van dit hoofdstuk is dat bIAP toediening in een acuut myocard infarct model in muizen de productie van mMCP-1 chymase door mestcellen vermindert en de inflammatoire reactie vermindert. Dit zou een nieuwe rol voor bIAP kunnen impliceren in het verminderen van cardiogene shock na een acuut myocard infarct.

Tijdens hartchirurgie met gebruik van de hartlong machine ontstaat een verminderde doorbloeding van de darm. Een gevolg hiervan is het vrijkomen van endotoxine in de circulatie. In **hoofdstuk 4** wordt de APPIRED studie (Alkaline Phosphatase in Prevention of Ischemia Reperfusion Damage) beschreven. In een dubbel blind, gerandomiseerde, placebo gecontroleerde studie werden 63 patiënten, die een electieve CABG moesten ondergaan, geïncludeerd. Bovine intestinaal alkalische fosfatase of placebo werd intraveneus toegediend als een bolus net voor het starten van de anaesthesie gevolgd door een continu infuus gedurende 36 uur. Het primaire eindpunt was de reductie van de inflammatoire reactie. Daarnaast werden

klinische parameters bestudeerd. De inflammatoire reactie als gevolg van CABG met gebruik van de hartlong machine was laag in zowel de bIAP- als de placebo groep. Vijf patiënten in de placebo groep lieten een significante stijging van TNFα zien, die werd gevolgd door een significante stijging van IL-6 en IL-8. Een dergelijk TNFα stijging werd niet gezien in de bIAP groep, hetgeen de anti-inflammatoire werking van bIAP bevestigt. De anti-inflammatoire cytokines IL-10, IL-2 en IL-4 werden niet beïnvloed door de toediening van bIAP. Andere factoren representatief voor inflammatie zoals C-Reactive Protein (CRP) waren niet significant verschillend tussen de twee groepen. Er werd geen verschil gezien in klinische parameters. De intraveneuze toediening van bIAP als een bolus gevolgd door continu infuus leidt tot een initiële stijging van het alkalische fosfatase als gevolg van de toediening van de bolus gevolgd door een significante stijging van alkalische fosfatase na 4 tot 6 uur na het starten van de operatie. Dit fenomeen van het vrijkomen van endogene alkalische fosfatase wordt beschreven in **hoofdstuk 5**. De enzymactiviteit van het alkalische fosfatase in de tweede piek kon geremd worden door L-homo arginine (LHA), waaruit geconcludeerd wordt dat de tweede piek alkalische fosfatase veroorzaakt wordt door het vrijkomen van niet-weefsel specifieke alkalische fosfatase. Door middel van isoenzym analyse werd uitgesloten dat het om bot type alkalische fosfatase ging. De meest waarschijnlijke bron van dit endogene alkalische fosfatase zal dus lever type alkalische fosfatase zijn. Dit endogene alkalische fosfatase, dat alleen gezien werd bij toediening van bIAP als een bolus gevolgd door een continu infuus, zou een rol kunnen spelen in het immuun systeem en is voor zover bekend nooit eerder beschreven. Tijdens de APPIRED studie was in alle hartlong machines laag gedoseerd aprotinine onderdeel van de prime vloeistof. Tegenwoordig is het gebruik van aprotinine niet meer toegestaan vanwege patiënt veiligheids aspecten. Dat aprotinine mogelijk de uitlokkende factor in het vrijkomen van endogene alkalische phophatase kan zijn is onderzocht in **hoofdstuk 6**. In dit hoofdstuk wordt een kleine studie beschreven bij patiënten die een electieve CABG ondergaan zonder gebruik van aprotinine. Bovendien worden twee verschillende doseringen van bIAP toegepast. BIAP werd toegediend als bolus, als bolus gevolgd door continue infuus. Een controle groep ontving placebo. Uit deze kleine studie blijkt dat aprotinine niet de uitlokkende factor is voor het vrijkomen van endogene alkalische phosphatase, en dat alleen een bolus bIAP gevolgd door continue infuus endogene alkalische phosphatase induceert. Het meest intrigerend van het vrijkomen van endogeen alkalische fosfatase is het feit dat het alleen gezien werd na toediening van bIAP als een bolus gevolgd door continu infuus. Dit heeft geleid tot de experimenten in varkens waarvan de resultaten worden beschreven in **hoofdstuk 7**. In deze experimenten werd bIAP intraveneus, intramusculair en subcutaan toegediend zowel een enkele bolus als meerdere bolussen verdeeld over 3 dagen en werden de verschillen in farmacokinetiek beschreven. Echter, in geen van de experimenten bij varkens kon endogeen alkalische phosphatase worden geïnduceerd.
Het fenomeen van de geïnduceerde endogene alklaische fosfatase bij patiënten die

een electieve CABG ondergaan met het gebruik van de hart long machine en die een bolus bIAP gevolg door continu infuus krijgen toegediend is uniek en tot dusver niet eerder beschreven.
Alkalische fosfatase was tot nu toe bekend als 'schade enzym'. Het is echter aannemelijk dat als alkalische fosfatase in het bloed stijgt dit een reactie van ons lichaam is op een 'bedreiging' van buitenaf, bijvoorbeeld een ontsteking. Alkalische fosfatase zou kunnen worden beschouwd als acute fase eiwit en waarbij een hoog alkalische fosfatase gehalte beschermend zou kunnen zijn tegen ontstekingsreacties. De plasma spiegel van preoperatief alkalische fosfatase zou een voorspelling kunnen geven over morbiditeit en mortaliteit op een zelfde manier als dit beschreven is voor anti-endotoxine in het lichaam. Patiënten zouden beschermd kunnen worden door toediening van alkalische fosfatase en door inductie van endogene alkalische fosfatase.

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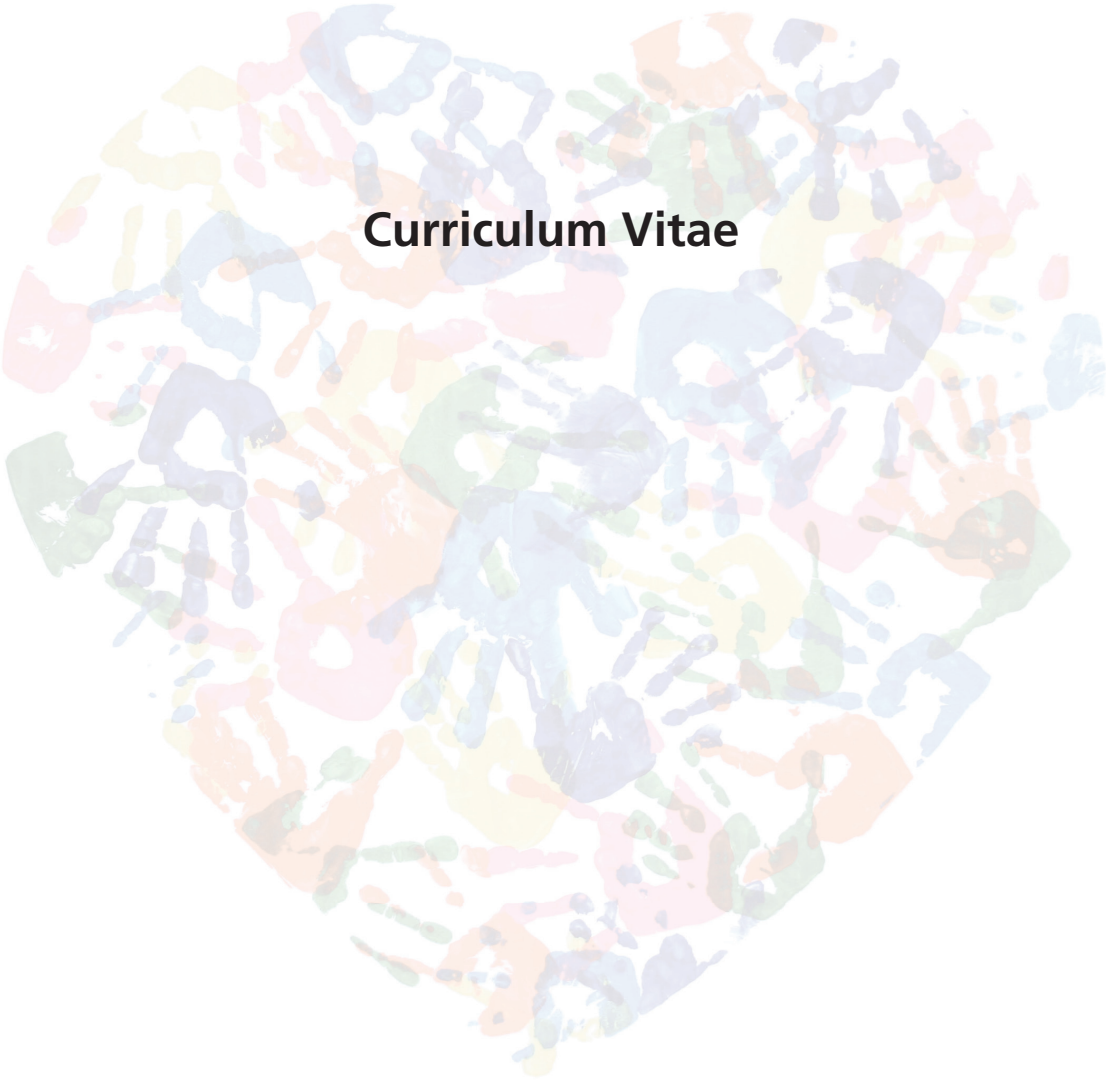

Curriculum Vitae

Suzanne Kats is geboren op 25 mei 1973 in Veendam. In 1991 behaalde zij haar diploma aan het Christelijk Gymnasium in Utrecht. Hierna studeerde zij geneeskunde aan de Erasmus Universiteit in Rotterdam waar zij in 1998 het artsexamen aflegde.
Na wat omzwervingen, begon zij in 2001 als arts assistent cardiothoracale chirugie in het UMC Utrecht, waar zij in 2002 werd aangenomen door prof. dr. A. Brutel de la Rivière voor de opleiding tot cardiothoracaal chirurg. Haar vooropleiding deed zij met veel plezier in het Albert Schweitzer Ziekenhuis in Dordrecht. Na haar vooropleiding kon zij haar vervolgopleiding niet voortzetten in het UMC Utrecht in verband met het tijdelijk beëindigen van de opleiding cardiothoracale chirurgie. In het Catharina Ziekenhuis Eindhoven vervolgde zij haar opleiding tot cardiothoracaal chirurg. Haar opleiders waren prof.dr. O.C.K.M. Penn, dr. J.P.A.M. Schönberger en dr. E. Berreklouw. In 2009 werd de opleiding tot cardiothoracaal chirurg afgerond. Tijdens haar opleiding en erna werkte zij aan haar promotieonderzoek. Na haar opleiding werkte zij als chef de clinique in het Antonius Ziekenhuis in Nieuwegein. Sinds september 2010 werkt zij als cardiothoracaal chirurg in het MUMC Maastricht en vanaf 1 september 2011 is zij daar opgenomen in de staf van prof. dr. J.G. Maessen.

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