



## DOCTORAL THESIS SUMMARY

### **Monomeric C-reactive protein, macrophage activation, and vascular disease pathology**

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#### **Background**

C-reactive protein (CRP) is an acute phase pentameric protein produced by the liver in response to inflammatory stimuli and consequent increase in IL-6 levels. Native CRP (nCRP) dissociates in five free monomers (mCRP) at site of inflammation, by interacting with phosphocholine (PC) groups present on activated cell membranes. The main bioactive properties associated with mCRP are induction of monocytes recruitment and their adherence to endothelium, activation of the inflammatory macrophage phenotype (M1) and their absorption of ox-LDL, as well as upregulation of MCP-1, ICAM-1, VCAM-1, ELAM-1, IL-8. Therefore, consequences of mCRP biological activity are represented in its disease modifying behaviour, particularly in the vascular components of cardiovascular and neurodegenerative diseases. The recently characterized CRP dissociation inhibitor C10M has shown promising results in blocking the effects of administered CRP in rat models, therefore it is contemplated as a therapeutic avenue to abolish mCRP production and its subsequent noxious sequelae.

#### **Study 1- mCRP caused aggregation of monocytes and polarization to M1, in a FAK-dependent fashion, which was partially inhibited by C10M**

This study aimed to explore the FAK-dependent mCRP modulation of monocyte attachment and clustering, concurrent with differentiation into M1 macrophage sub-populations. Furthermore, the ability of the mCRP inhibitor C10M to halt this process and possibly prevent mCRP-induced release of pro-inflammatory cytokines, was investigated. Thus, U937 cell line and human PBMs, extracted from two healthy volunteers, have been exposed to mCRP, and the use of a specific FAK inhibitor and C10M have been implemented to test variations in the aforementioned processes. The main employed techniques were microscopy, confocal analysis, FACS analysis, Western blotting, and ELISA.

#### *Originality:*

- mCRP, but not nCRP nor LPS, induced monocyte aggregation in vitro, over a timeframe of 3–24 h. This occurred concurrently with augmented p-FAK expression and perinuclear to nuclear translocation, which could be completely abrogated by the inhibitor C10M. Furthermore, mCRP-induced monocyte aggregation was associated with M1 polarization of human PBMs, and increased secretion of pro-inflammatory cytokines IL-8, and IL-1 $\beta$ . Pre-incubation with the FAK inhibitor I prior to treatment with mCRP abolished monocyte aggregation, indicating a FAK-dependent mechanism. This represents the first work to show a direct link between mCRP, FAK, and monocyte aggregation, and deriving polarization to the pro-inflammatory phenotype. C10M reduced iNOS, the mean cellular FAK expression, whilst blocking nuclear localization, and partially reduced the expression of markers associated with M1 macrophage differentiation.



- Acute exposure of primary PBMs to mCRP caused a reduction in phosphorylation of MAP kinase pathways ERK-1 and JNK 1/2. The C10M inhibitor did not further modulate these cells signaling pathways.
- mCRP increased phosphorylation of p53. This indicates its potential pro-apoptotic role. C10M exacerbated phospho-p-53 expression, which would need to be taken into account if this small molecule inhibitor was to be considered suitable for therapeutic use.

### **Study 2- C10M inhibited brain vascular expression of mCRP after intraperitoneal injections, in a dyslipidemia ApoE<sup>-/-</sup> murine model**

The objective of this study was to investigate the capacity of intraperitoneally (IP) injected mCRP to traverse the blood-brain barrier (BBB), its localization within the brain parenchyma, and the ability of C10M to prevent against its accumulation in order to comprehend the dynamics of mCRP vascular distribution linked with possible neurodegenerative consequences. Hence, 24 ApoE<sup>-/-</sup> on a high fat protocol have been categorized in three groups, namely control, exclusively mCRP receiving group, and mCRP plus C10M receiving group. After euthanasia, their brain parenchyma and microvasculature were investigated for mCRP deposition and CD105 expression, using histological analysis and immunohistochemistry.

#### *Originality:*

- Multiple IP injections of mCRP over four weeks into young ApoE<sup>-/-</sup> mice (70μg/Kg, twice a week), concomitant with a high-fat diet, led to a significant transfer of mCRP into the brain parenchyma, and its expression within microvessels and neurons of the hippocampus, cerebral cortex, corpus callosum, mesencephalon, and metathalamus. mCRP transferred to the brain microvasculature via systemic induction of inflammation, and subsequent increase of the BBB permeability. Within the brain parenchyma, the mCRP deposited within the microvessels led to disruption of the endothelial cell (EC) junctions, leakage into the extracellular matrix, and following uptake by local neurons.
- mCRP was strongly co-localized with CD105 in the lumen and in the intimal ECs of cortical microvessels in serial sections of mice who were injected with mCRP but not in control group. This points to mCRP-induced angiogenic activation of the vessels, their increased permeability and susceptibility to inflammatory insult, leakage, and degeneration.
- The C10M inhibitor completely abrogated mCRP deposition within the brain, in mice treated with both this C10M and mCRP, and consequently CD105 expression. Direct binding of C10M to mCRP decreased its ability to induce systemic inflammation necessary for BBB disruption and, thus, halting its entrance into the brain parenchyma.

### **Conclusions**

Preserving the EC barrier from mCRP-associated build-up of immune cell or platelet aggregates represents a possible therapeutic protection against vessel stenosis and thrombosis, since focalized inflammatory activity aggravates vascular damage, and intimal EC penetration marks the early stages of atherosclerotic plaque development. By its deposition in the brain microvessels, disturbance of the EC architecture, and consequent spreading in the surrounding cerebral neurons, mCRP may cause dysfunctions of the neurovascular unit. Administration of the new small molecule inhibitor C10M, which in this work showed efficacy in totally abolishing accumulation of mCRP and expression of CD105 within the mice brain parenchyma and microvasculature, could help to prevent this pathological process. To evaluate the possible use of C10M in prevention or blocking of neurodeterioration, more research including studies involving human subjects/clinical trial is required.