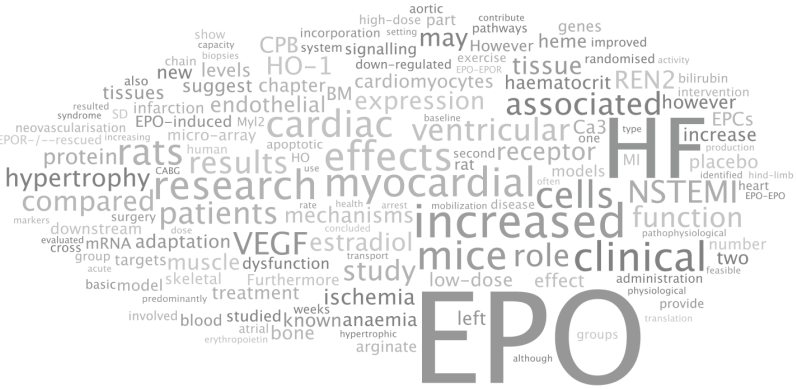
9 CHAPTER

Summary and future perspectives



SUMMARY

Heart failure (HF) is a clinical syndrome, involving not only the heart, but many other vital organs as well, including the brain, muscles, kidney and bone marrow.[1] HF is associated with an increased serious risk of hospitalisation and mortality, and the economic burden of HF for health care systems and society is enormous.[2] Atherosclerotic coronary disease is an important risk factor for the development of HF. New treatment options to preserve left ventricular function after myocardial infarction are therefore warranted. Furthermore, by increasing our understanding of the pathophysiological processes involved in HF, we may be able to improve pharmacological treatment options. Therefore, we studied the extra-haematopoietic mechanisms of erythropoietin (EPO) and the EPO-EPO receptor system in HF. Furthermore, we studied downstream pathways of erythropoietin and potential targets for intervention, to gain further insight in the pathophysiological processes involved in HF.

Extra-haematopoietic mechanisms of EPO and the EPO-EPO Receptor system

In the first part of this thesis, we explored the extra-haematopoietic mechanisms of EPO and the role of the EPO-EPO receptor system in HF. We intended to delineate the mechanisms of EPO-induced cardiac vascular endothelial growth factor (VEGF) production. Furthermore, we established if VEGF is crucial for EPO-induced improvement of cardiac performance (**chapter 3**). We studied the effects of EPO on VEGF expression in cultured endothelial cells and cardiomyocytes and EPO-treated hearts. The role of VEGF in EPO-induced neovascularisation was studied with two distinct VEGF-neutralising antibodies (or irrelevant control IgG) in an aortic sprouting assay and in rats with HF after myocardial infarction (MI) treated with EPO. EPO stimulated VEGF mRNA expression through the STAT3 signal transduction pathway in neonatal rat cardiomyocytes, but not in endothelial cells or fibroblasts. Similarly, the direct effects of EPO on endothelial sprouting were modest and VEGF independent. In rats with HF, EPO increased VEGF protein expression predominantly in cardiomyocytes, associated with a 37% increase in capillary density and improved cardiac performance. Administration of VEGF-neutralising antibodies abrogated the salutary effects of EPO on myocardial microvascularisation and function. VEGF neutralisation attenuated EPO-induced proliferation of myocardial endothelial cells and reduced myocardial incorporation of endothelial progenitor cells (EPCs) in rats with alkaline phosphatase-labelled bone marrow cells. We conclude that VEGF is crucial for EPO-induced improvement of cardiac function in HF. EPO fosters VEGF expression predominantly in cardiomyocytes, which in turn stimulates myocardial endothelial proliferation and incorporation of EPCs.

The role of the EPO receptor in physiologic hypertrophy was studied next (**chapter 4**). We have demonstrated that EPO via its receptor exerts cardioprotective effects in pathophysiological hypertrophy (e.g. MI and HF).[3,4] It is unknown however if the EPO-EPOR signalling pathway exerts similar results in physiological hypertrophy. EPO receptor rescued knock-out mice ($EPOR^{-/-}$-rescued mice) are mice that express the EPO receptor exclusively in the haematopoietic cells.[5] These mice have accelerated maladaptive remodelling upon various stimuli, because of an insufficient angiogenic response.[6-9] Mice had unlimited access to a running wheel for

four weeks. We show that EPOR$^{-/-}$-rescued mice have a reduced exercise capacity, which is associated with an impaired adaptation of cardiac and skeletal muscle. The increased exercise capacity seen in WT mice resulted in a larger cardiac adaptation in HW in the WT-ex mice (26%) as compared to the EPOR-ex mice (10%). This increased cardiac adaptation was accompanied by myocyte hypertrophy and neovascularisation as shown with the increase in cross sectional area and capillary-to-myocyte ratio in both cardiomyocytes as skeletal muscle. EPOR$^{-/-}$-rescued did not show this adaptation to increased workload. Furthermore, exercised WT mice show a shift in muscle fibre type to adapt to the increased workload (type II to type I), where EPOR$^{-/-}$-rescued mice do not show this adaptation al all. We concluded that the absence of EPO-EPOR signalling leads to a lesser exercise capacity upon voluntary wheel-running. We suggest that EPO-EPOR signalling is important for physiological adaptation during exercise.

Studies in animal models of acute myocardial infarction suggest that ischemia-reperfusion (I/R) injury accounts for up to 50% of the final size of an MI, and in I/R models, a number of strategies have been shown to ameliorate lethal reperfusion injury.[10] Of note, the translation of these beneficial effects into the clinical setting has been disappointing.[11] To corroborate our data we collected in animal studies, we evaluated a human model of I/R injury and compared the rate of apoptosis after cardiopulmonary bypass (CPB) and cardioplegic arrest during coronary artery bypass grafting (CABG) surgery between atrial and ventricular tissue (**chapter 5**). During CABG surgery with CPB and cardioplegic arrest, sequential biopsies were taken from the right atrial appendage and left ventricular anterior wall before CPB and after aortic cross clamp release. We assessed the change in number of apoptotic cells and biochemical markers of myocardial ischemia and renal dysfunction. CPB was associated with a transient small, but significant increase in CK, CK-MB, troponin-T and NT-proBNP levels. A higher number of apoptotic cells as assessed by caspase-3 staining was found in the ventricular biopsies taken after aortic cross clamp release compared with the biopsies taken before CPB. The number of apoptotic cells in the atrial appendage was not altered during CPB. Correlation between the duration of aortic cross clamp time and the change in caspase-3 positive cells in the left ventricular wall was of borderline significance. We concluded that CABG surgery with CPB and cardioplegic arrest is associated with an elevated rate of apoptosis predominantly in ventricular tissue. Ventricular tissue may be more sensitive to detect changes than atrial tissue, and may be more useful to investigate the protective effects of therapeutic intervention.

Downstream pathways of erythropoietin: feasible targets for intervention

In the second part of this thesis, we focused on feasible targets for intervention to ameliorate the effects of ischemia in the heart. In **chapter 6**, we investigated whether administration of estradiol to male mice augments mobilisation of bone marrow-derived EPC and incorporation into foci of neovascularisation after hind-limb ischemia, thereby contributing to blood flow restoration. EPC culture assay performed two weeks after estradiol pellet implantation revealed a significantly greater number of circulating EPCs in the high-dose estradiol group than in the low-dose estradiol and placebo groups. Three and four weeks after induction of hind limb ischemia, perfusion was significantly greater in high-dose estradiol mice than in low-dose estradiol or placebo treated mice. At one and four weeks after hind-limb ischemia

surgery, more bone marrow-derived EPCs, identified as β-galactosidase-positive cells, were observed in ischemic regions from high-dose estradiol animals than in low-dose or placebo groups. These results indicate that estradiol dose-dependently increases the levels of EPCs in peripheral blood in male animals, improves the recovery of blood flow, and decreases limb necrosis after hind-limb ischemia, and that this enhancement occurs, in part, through augmentation of EPC mobilisation and greater incorporation of bone marrow-derived EPCs into foci of neovascularisation.

I/R-injury, for example in acute myocardial infarction, is accompanied by an severely increased production of reactive oxygen species and subsequent cell death, contributing to the loss of viable myocardial tissue after sustained MI.[10] In **chapter 7** we evaluated the safety and efficacy of an exogenous heme oxygenase (HO)-1 inducer (heme arginate) in patients with a non-ST-elevation myocardial infarction (NSTEMI) to scavenge these reactive oxygen species. Degradation products of HO-1 activity have been suggested to act as anti-oxidants.[12-15] The inducible isoform of HO, named HO-1, can be induced in virtually all tissues and represents the rate limiting step in the degradation of the pro-oxidant heme molecule into biliverdin, carbon monoxide (CO), and iron.[12] Biliverdin is then converted by biliverdin reductase (BVR) into bilirubin, whereas iron induces the expression of the iron sequestering molecule ferritin.[12,16-18] At physiological concentrations, bilirubin, CO and ferritin all exhibit high ant-oxidant and cytoprotective properties.[18-21] In our study, patients with NSTEMI were open label randomised to a single dose of heme arginate or placebo. Heme arginate or placebo was infused within three hours after hospital admission for NSTEMI. A single high dose of heme arginate after NSTEMI did not increase plasma ferritin and total, direct and indirect bilirubin concentrations, HO-1 mRNA expression and HO protein activity in peripheral blood mononuclear cells as compared to the placebo group. Safety parameters were all comparable between the two groups. However, NSTEMI itself did activate the HO-1 system. Median total bilirubin levels were increased with 60% after NSTEMI. Also, median HO-1 mRNA (17.5 times compared to baseline) and median HO protein activity (780% compared to baseline) were increased after NSTEMI. We concluded from this study that although there is an endogenous HO-1 response to NSTEMI, adding an exogenous HO-1 inducer does not increase downstream HO-1 markers further, however, heme arginate administration is safe in patients with NSTEMI. We suggest that before embarking on larger sized trials, a dose-finding study should be carried out, in order to determine the optimal dose for patients with MI (NSTEMI of STEMI).

One of the common co-morbidities in HF Is anaemia, which presence is associated with a particularly poor outcome.[22-24] The causes of anaemia in HF are only partially understood, although several mechanisms have been implicated.[25-29] Because bone marrow dysfunction may contribute to HF-associated anaemia, we investigated mechanisms of BM dysfunction in an established model of HF (**chapter 8**), the transgenic REN2 rat, which is characterized by severe hypertrophy and ventricular dilatation and Sprague Dawley (SD) rats as controls. Secondly, we investigated whether stimulation of haematopoiesis with EPO could restore anaemia and BM dysfunction. Number of burst forming units-erythroid (BFU-E) was severely decreased in BM of REN2 rats compared to SD rats. EPO treatment increased haematocrit in the SD-EPO group, however in the mildly anaemic REN2 rats, there was no effect. This was paralleled by a 67%

decrease in BFU-E in BM of REN2 rats compared to SD. EPO significantly improved BFU-E in both SD and REN2, but could not restore this to control levels in the REN2 rats. Expression of several genes involved in differentiation and mobilisation of the BM is deregulated in REN2 rats, and EPO did not normalise this. Altogether, these results suggest that BM dysfunction is an important contributor to HF-associated anaemia in the hypertensive REN2 rat and that EPO is not an effective agent to treat HF-associated anaemia, at least in this model.

TRANSLATIONAL RESEARCH

Before discussing what future experiments could help to unravel the pathways of EPO and the potential clinical applicability of EPO or its derivatives, I would like to share some thoughts on translational research.

To improve human health, scientific discoveries must be translated into practical applications. Such discoveries typically begin at 'the bench' with basic research - in which scientists study disease at a molecular or cellular level - then progress to the clinical level, or the patient's 'bedside.' Often, preclinical researchers are doing the 'why and how', where clinical researchers are looking for the best ways to treat their patients. Knock-out and overexpression models are used to unravel the mechanisms behind diseases. However, this approach in the human setting is clearly not feasible. Scientists are however increasingly aware that this bench-to-bedside approach to translational research is really a two-way street. Basic scientists provide clinicians with new tools for use in patients and for assessment of their impact, and clinical researchers make novel observations about the nature and progression of disease that often stimulate basic investigations. Translational research has proven to be a powerful process that drives the clinical research engine. Unfortunately, as is common in translation research, results found in experimental models, are not necessarily translated to the human setting. Preclinical and clinical research are two distinct worlds and often there is a disappointing consistency between these two worlds. Growing barriers between clinical and basic research, along with the ever the increasing complexities involved in conducting clinical research, are making it more difficult to translate new knowledge to the clinic - and back again to the bench.

However, a stronger research infrastructure could strengthen and accelerate this critical part of the clinical research enterprise. Of pivotal importance is the doctor-scientist (MD, PhD), who is able to bridge the gap between basic and clinical science. Their increasing awareness that not only the 'why and how' is of major importance, but also the feasibility of these new findings for their patients should bring health care forward.

FUTURE PERSPECTIVES

Now the results of the HEBE III are published,[30] two more large-scale randomised EPO trials still have to reveal their results. First, the results of second study evaluating the effect of EPO in first ST-segment elevation MI (REVEAL) are awaited in the fourth quartile of 2010.[31] Second, the results of first large randomised study to evaluate the effects of EPO in HF (RED-HF) are awaited in 2011.[32] Results of both should give a definitive answer on the use of EPO in cardiovascular disease. However, regardless of these results, the search for cellular protection against ischemia and I/R-injury continues.

The dosing regimens used in previous HF studies, all resulted in a significant increase in haematocrit levels. When applied to the clinical situation, this could lead to hypertension, seizures, vascular thrombosis and death, possibly related to abruptly increased haematocrit levels.[33] Therefore, our group recently evaluated the effect of a low-dose EPO bolus that had no effect on haematocrit. Similar to high-dose EPO, low-dose treatment resulted in statistically improved cardiac function and improved myocardial microvascularisation, although the effect was slightly less pronounced.[3] These results do not only suggest that the beneficial effects of EPO on the heart may be independent of an increased haematocrit but also suggest that low-dose EPO might provide a safe and effective strategy in patients.

Another option to avoid the potentially negative effects of chronic EPO therapy in haematocrit values could be the use of recently discovered carbamylated EPO,[34-39] and other non-

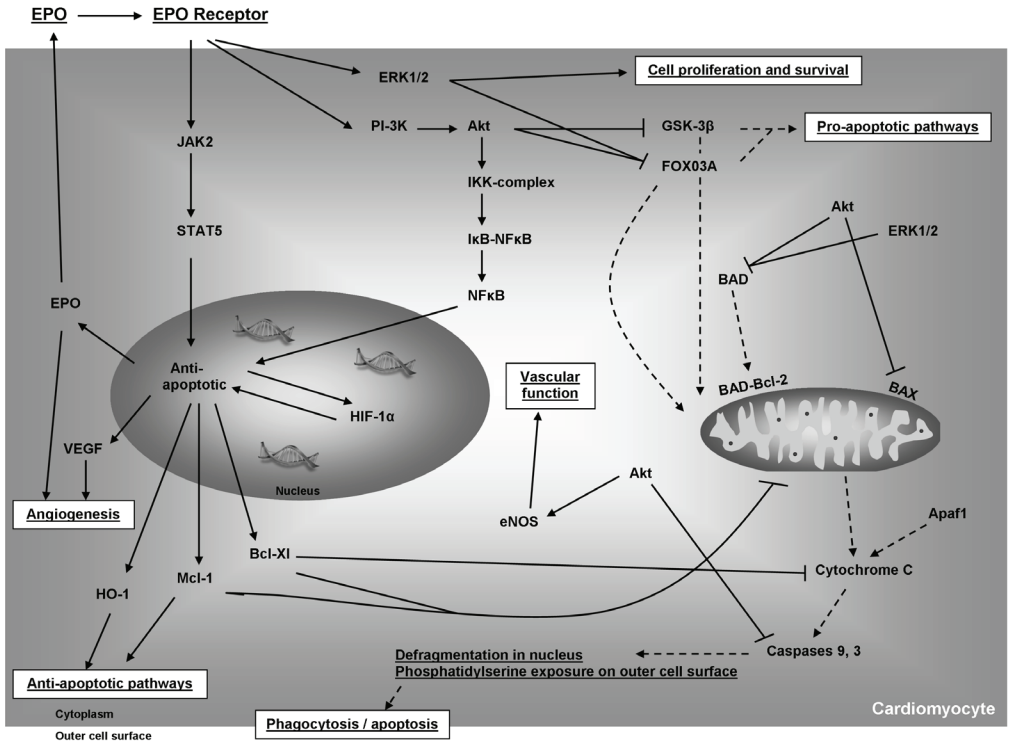


Figure 1. Overview of the potential mechanisms of cytoprotection of erythropoietin (EPO). Activation of the EPO receptor by EPO leads to downstream activation of intracellular pathways, such as PI3K/akt, ERK1/2, and STAT. These pathways originate with the binding of EPO to the EPO receptor to activate JAK2. Subsequently, PI3K and Akt are activated by downstream phosphorylation. Activation of STAT can regulate EPO-mediated cell protection and protect against apoptosis. EPO maintains cellular integrity and prevents apoptosis through a number of pathways, such as the modulation of apoptosis protease activating factor-1, the release of cytochrome c, and the prevention of activation of caspases 9 and 3. Dotted lines, suppressed pathways; solid lines, activated pathways. For colour figure, see supplement 1.

Table 1. Baseline characteristics

	Con-PL (n=5)	Con-EPO (n=4)	MI-PL (n=5)	MI-EPO (n=5)
BW (gr)	359±7	433±22	397±9	403±9
HW (mg)	1181±37	1650±172*	1959±297*	1598±89*
HW/BW (mg/gr)	3.28±0.11	3.82±0.23	4.91±0.70*	3.97±0.20*‡
MAP (mmHg)	90.8±3.8	97.0±3.1	91.0±4.5	89.6±2.9
HR (bpm)	384±36	321±13	325±13	333±9
dPdtmax (mmHg/s)	9900±764	10556±976	6225±1126*	9518±247
dPdtmin (mmHg/s)	-10775±462	-9753±743	-5737±830*	-8456±274
LVEDP (mmHg)	2.8±0.5	2.7±1.8	19.8±7.1*	12.8±2.5*‡
Ht (%)	51±1.9	63±5.2*	47±1.9	59±3.5*‡
ANP (fold change)	1.00±0.12	1.78±0.40	7.65±1.23*	5.17±1.06*‡

Table shows the baseline characteristics of the groups used in the micro-array. Sprague Dawley rats were used. HF was induced by permanent ligation of the left ascending coronary artery to produce a myocardial infarction (MI) and control rats received a sham procedure. Three weeks after MI, rats were randomly assigned to treatment with the long-acting erythropoietin darbepoetin alfa (EPO, 40 μg/kg) or placebo (PL), given once every three weeks subcutaneous. Con = control rats, BW = body weight, HW = heart weight, MAP = mean arterial pressure, HR = heart rate, dPdtmax and dPdtmin are indices of maximal contraction and relaxation, LVEDP = left ventricular end diastolic pressure, Ht = haematocrit. To determine if true heart failure existed, we measured atrial natriuretic peptide (ANP, shown as fold change vs. SD). Data is presented as mean±SEM. *p<0.05 vs. Con-PL, ‡ p<0.05 vs. MI-PL

erythropoietic derivates of EPO,[40,41] retaining the tissue protective properties, without the undesired effect on erythropoiesis.[42] Two independent studies have demonstrated that these non-erythropoietic EPOs retain their cardioprotective potential in models of acute MI.[43,44] It is however uncertain whether these new EPOs will improve cardiac function in chronic HF. Large randomised trials have to be conducted to answer this question.

Another possibility is to better understand downstream EPO signalling (figure 1). We conducted a micro-array to elucidate the transcriptomic changes that occur after EPO treatment in post-MI HF. For this micro-array, we applied ventricular hypertrophic mRNA from tissue obtained from a post-MI HF model as previous described.[4,45,46] A micro-array is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, known as probes. Probe-target hybridisation is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labelled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a micro-array experiment can accomplish many genetic tests in parallel, in our study, the entire genome of a rat. We attempted to identify a set of key genes which are differentially regulated by EPO and can provide us with new possible targets for research.

Table 1 shows the baseline characteristics of the groups that were analysed with the micro-array technique. Among other things, we compared the transcriptome of rats that had ischemic HF

by permanent ligation of the left ascending coronary artery vs. rats with ischemic HF treated with EPO. Seven genes were identified that were up- or downregulated after EPO treatment.

Upregulated genes

Upregulated during skeletal muscle growth 5 (Usmg5), also known as diabetes-associated protein in insulin-sensitive tissues (Dapit), is a protein that is expressed in skeletal muscle. One study found it is downregulated in insulin-sensitive tissues in streptozotocin-induced diabetes.[47] It is also associated with ATP synthase in heart mitochondria.[48,49] Little is known about the exact function of Usmg5. Upregulation in response to active stretching of skeletal muscle and downregulation of Dapit mRNA in insulin-sensitive rat tissues upon induction of streptozotocin-induced diabetes may point to a role in the energy metabolism of cells, in glucose metabolism, and/or in oxidative phosphorylation.

The second upregulated gene, myosin regulatory light chain 2, ventricular/cardiac muscle isoform, or Myl2, encodes the regulatory light chain associated with cardiac myosin beta heavy chain. Calcium triggers the phosphorylation of regulatory light chain that in turn triggers contraction. Mutations in Myl2 are associated with left ventricular hypertrophic cardiomyopathy.[50-53] Upregulation by EPO could mean that Myl2 has a role in preventing left ventricular hypertrophy, however not much is known about the role of Myl2 in HF.

Downregulated genes

Carbonic anhydrase 3 (Ca3) is part of a family of enzymes that catalyze the rapid conversion of CO2 and H2O to bicarbonate and protons, a reaction that occurs rather slowly in the absence of a catalyst. The primary function of Ca3 in animals is to maintain acid-base balance in blood and other tissues, and to help transport CO2 out of tissues. Only one study describes a role for Ca3 in the emergence of cardiac hypertrophy.[54] Apparently, activation of the plasma membrane Na^+-H^+ exchanger (NHE1) and Cl^- -$HCO3^-$ exchanger (AE3) has emerged as a central point in the hypertrophic cascade. Both NHE1 and AE3 bind carbonic anhydrase (Ca3), which activates their transport flux, by providing H^+ and $HCO3^-$, their respective transport substrates. Treatment of cardiomyocytes *in vitro* with phenylephrine to induce hypertrophy, increased the expression of Ca3, where a specific inhibitor of Ca3 prevented the phenylephrine-induced hypertrophy. Possibly, the expression of Ca3 is downregulated in our *in vivo* model as a protective mechanism.

The second downregulated gene, Eukaryotic translation elongation factor 1 alpha 1, Eef1α1, encodes a ribosomal protein and plays a role in inflammation. For example, it is identified as an auto-antigen in 66% of patients with Felty syndrome, a syndrome characterised by the combination of rheumatoid arthritis, splenomegaly and neutropenia. The role of Eef1α1 in HF is not known and calls for speculation. HF is often characterised by increases in circulating pro-inflammatory cytokines (TNFα, interleukin (IL)-6, IL-1, and IL-2) and their soluble receptor or receptor antagonists that become more pronounced as myocardial function deteriorates.[55-60] In addition, increased production of pro-inflammatory cytokines and other inflammatory markers may identify patients at increased risk of developing HF in the future.[61,62] Furthermore, a cause-and-effect relationship may contribute to the development of HF, as excess TNFα and IL-6 have a deleterious effects on cardiac function. Eef1α1 might play a role in the downstream effects of inflammatory cytokines.

Some limitations to this approach apply. First, we do not see any upregulation of known factors associated with the apoptotic and neovascularising properties of EPO, such as STAT3 and VEGF.[8,45,63,64] However, one of the limitations of a micro-array is that the association with protein levels remains unknown. Second, the tissue was harvested one week after the last administration of EPO. We cannot rule out that the effects of this last administration are not picked up at a transcriptional level.
Nevertheless, these factors provide additional insight in the downstream pathways of EPO signalling and could contribute towards new therapeutic possibilities.

Table 2. Up- and downregulated genes in MI compared to MI-EPO

Upregulated			
Description	**Fold change**	**p**	**Symbol**
Upregulated during skeletal muscle growth 5	2,4	0,03	Usmg5
Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	1,7	0,03	Myl2
Ribosomal protein L22 like 1	1,5	0,04	RPL22L1
Thioesterase superfamily member 2	1,5	0,04	Them2
Downregulated			
Description	**Fold change**	**p-value**	**Symbol**
Carbonic anhydrase 3	8,1	0,02	Ca3
Eukaryotic translation elongation factor 1 alpha 1	1,6	0,01	Eef1a1
WW domain binding protein 5	1,5	0,003	Wbp5

Table describes the genes found in the micro-array. Depicted are the genes found comparing MI vs. MI-EPO, >1.5 fold change, $p<0.05$.

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